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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our laboratory has recently identified a new protein, Pin1, that is involved in checkpoint control. Pin1 interacts with mitotic phosphoproteins and helps to orchestrate the timing of mitotic events. We found that Pin1 is highly overexpressed in breast cancer. Pin1 levels correlate with the levels of cyclin D1 protein as well as with cyclin D1 mRNA levels. We have shown that Pin1 is a transcriptional acitvator of cyclin D1. Activation occurrs indirectly through the binding of phosphorylated c-jun. Our data indicate that Pin1 may contribute to neoplastic transformation by causing accumulation of cyclin D1.

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I. Introduction

Cell division is regulated by several control points termed checkpoints. These checkpoints are believed to be regulated by protein phosphorylation/dephosphorylation cascades. Our laboratory has recently identified a new protein, Pin1, involved in mitotic checkpoint control. Pin1 is a novel and conserved peptidyl-prolyl isomerase (PPIase) that is the first PPIase essential for cell growth and specifically isomerizes phosphorylated Ser/Thr-Pro bonds. By interacting with mitotic phosphoproteins, Pin1 helps to orchestrate the timing of mitotic events (1). The current study will help us to understand whether Pin1 is a candidate tumor marker for breast cancer and whether screening of a larger cohort of pathological specimen is warranted, and whether increased Pin1 expression is due to genetic mechanisms or to non-genetic regulatory mechanisms. From the *in vitro* experiments we will learn whether Pin1 has an oncogenic potential, and if so, which domain is responsible for their oncogenic potential and which are the interacting proteins.

II. Body

Task 1 (months 1 – 12)

To examine whether Pin1 or Pin2 are candidate tumor markers for breast cancer

It turned out that Pin1 levels correlate closely with the tumor grade and cyclin D1 levels in breast tumors. All the pertinent results are presented in the attached papers published in the EMBO Journal and Nature Cell Biology.

Pin2 levels in the 50 tumor specimen were significantly lower then in the 10 normal specimen examined, however there was no correlation with tumor grade, LN status of the patient, Cyclin D1 or PCNA or Estrogen Receptor Status. All the pertinent results are presented in the attached paper published in Oncogene.

Task 2 (months 12-24)

Several MCF7 cell lines inducible for Pin1 have been established. Inducibility has been verified by Western Blotting (see Fig. 3 of the attached manuscript).

For the in vivo investigation I choose a different approach since the techniques of generating transgenic mice have become readily available to us: An expression vector controlling Pin1 expression from the MMTV promoter was constructed, and injected blastocytes in our transgenic facility. We have now a number of transgenic Pin1 mice which we are currently screening for the expression of the Pin1 transgene. Subsequently, I will analyze the incidence of breast cancer in these mice.
In addition, we were able to obtain Pin1 knock-out mice from a Japanese group. I am currently performing breeding experiments with ras and neu transgenic mice. My question here is whether the Pin1 -/- background protects these mice from ras or neu induced breast cancers.

III. Key Research Accomplishments

- Examination of Pin1 levels in 50 primary breast cancer specimen
  Pin1 is overexpressed in 75% of breast cancers
- Correlation of Pin1 levels with clinicopathologic characteristics of the tumors
  Pin1 levels correlate with tumor grade and cyclin D1 levels
- Examination of the Pin1 interactions in vitro
  Pin1 is a transcriptional cotransactivator of the cyclin D1 promoter and interacts with c-jun.
- Pin1-transgenic mice were generated

- Examination of the Pin1-/- phenotype shows a striking resemblance with the cyclin D1 knock-out (manuscript in preparation)

IV. Reportable Outcomes

- Manuscripts: see attachment

1. Kishi S, **Wulf G,** Nakamura M and Lu KP. Telomeric Protein Pin2/TRF1 Induces Mitotic Arrest and Apoptosis in Cells with Short Telomeres and is Down-regulated in Human Breast Tumors. **Oncogene** 2001, 20:1497-1508

2. **Wulf G,** Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. **EMBO J**. 2001 20: 3459-3472

3. Ryo A, Nakamura M*, Wulf G*, Liou Y* and Lu KP. Prolyl isomerase Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. **Nature Cell Biology**, in press 7/2001
   * These authors contributed equally to this paper

Patent

Zhou XZ, **Wulf G** and Lu KP. Pin1 as a Marker for Abnormal Cell Growth. U.S. Patent No.60/167,800

V. Conclusions

- The prolylisomerase Pin1 may promote tumor growth through the accumulation of cyclin D1. Because of its unique enzymatic function Pin1 could serve as a target for inhibitory drugs.

VI. References

- see attached manuscript

The EMBO Journal Vol. 20 No. 13 pp. 3459–3472, 2001

Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1

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Phosphorylation on serines or threonines preceding proline (Ser/Thr-Pro) is a major signaling mechanism. The conformation of a subset of phosphorylated Ser/Thr-Pro motifs is regulated by the prolyl isomerase Pin1. Inhibition of Pin1 induces apoptosis and may also contribute to neuronal death in Alzheimer's disease. However, little is known about the role of Pin1 in cancer or in modulating transcription factor activity. Here we report that Pin1 is strikingly overexpressed in human breast cancers, and that its levels correlate with cyclin D1 levels in tumors. Overexpression of Pin1 increases cellular cyclin D1 protein and activates its promoter. Furthermore, Pin1 binds c-Jun that is phosphorylated on Ser63/73-Pro motifs by activated JNK or oncogenic Ras. Moreover, Pin1 cooperates with either activated Ras or JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter. Thus, Pin1 is up-regulated in human tumors and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. Given the crucial roles of Ras signaling and cyclin D1 overexpression in oncogenesis, our results suggest that overexpression of Pin1 may promote tumor growth.
Keywords: cancer/c-Jun/cyclin D1/Pin1/Ras signaling

Introduction

The reversible phosphorylation of proteins on serine/threonine residues preceding proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of various cellular processes, including cell division and transcription (reviewed by Hunter and Karin, 1992; Nurse, 1994; Nigg, 1995; Treisman, 1996; Whitmarsh and Davis, 1996; Karin *et al.*, 1997). For example, various growth factors and oncoproteins, such as oncogenic Ras, trigger a signaling cascade leading to the activation of c-Jun N-terminal kinases (JNKs), which phosphorylate c-Jun on $Ser^{63/73}$-Pro and enhance its transcriptional activity towards c-Jun target genes, including cyclin D1 (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Derijard *et al.*, 1994; Hinds *et al.*, 1994; Albanese *et al.*, 1995, 1999; Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Bakiri *et al.*, 2000). Overexpression of cyclin D1 often occurs in a variety of human cancers (Hunter and Pines, 1994), including ~50% of human breast tumors (Bartkova *et al.*, 1994; Gillett *et al.*, 1994; Lin *et al.*, 2000). Importantly, cyclin D1 can act as an oncogene that contributes to cell transformation by complementing a defective E1A oncogene (Hinds *et al.*, 1994). Conversely, inhibition of cyclin D1 expression causes growth arrest in tumor cells (Schrump *et al.*, 1996; Arber *et al.*, 1997; Driscoll *et al.*, 1997; Kornmann *et al.*, 1998). Moreover, knockout of cyclin D1 in mice blocks the proliferation of breast epithelial cells and retina, and inhibits tumor development in response to Ha-Ras (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Rodriguez-Puebla *et al.*, 1999). These results indicate that cyclin D1 plays an important role during oncogenesis, acting as a downstream mediator of Ras activity during tumor development, and that phosphorylation of c-Jun on $Ser^{63/73}$-Pro motifs is an important mechanism for the Ras-dependent up-regulation of cyclin D1. However, it is not clear whether the c-Jun activity is further regulated after Pro-directed phosphorylation.

Compelling evidence supports an additional and crucial signaling mechanism, which affects the state of Pro-directed phosphorylation sites, namely the conformational change induced by phosphorylation-specific prolyl isomerization. Such conformational change can regulate protein function (Zhou *et al.*, 1999). The phosphorylated Ser/Thr-Pro moiety exists in two distinct, slowly interconverting conformations: *cis* and *trans*. This conformational change introduces kinks into a peptide chain, thereby determining protein structure and function (Fischer, 1994; Galat and Metcalfe, 1995; Schmid, 1995; Hunter, 1998; Zhou *et al.*, 1999). Significantly, phosphorylation on Ser/Thr-Pro motifs further restrains the already slow *cis/trans* prolyl isomerization of peptide bonds (Yaffe *et al.*, 1997; Schutkowski *et al.*, 1998), and also renders them resistant to the catalytic action of conventional peptidyl-prolyl *cis/trans* isomerases (PPIases), including cyclophilins and FK506-binding proteins (Yaffe *et al.*, 1997). In contrast, Pin1 represents a new subfamily of highly conserved and phosphorylation-specific PPIases that isomerize only the phosphorylated Ser/Thr-Pro bonds, and not their non-phosphorylated counterparts (Yaffe *et al.*, 1997). Pin1 contains an N-terminal WW domain and a C-terminal PPIase domain (Lu *et al.*, 1996; Ranganathan *et al.*, 1997). The WW domain functions as a pSer/Thr-binding module, interacting with specific pSer/Thr-Pro motifs present in a defined subset of phosphoprotein substrates, including Cdc25C, tau, Myt1, S6 kinase, Rab4 and the C-terminal domain of RNA polymerase II (Lu *et al.*, 1999b). At the substrate, the PPIase domain of Pin1 isomerizes specific pSer/Thr-Pro bonds, and regulates protein function and dephosphoryl-

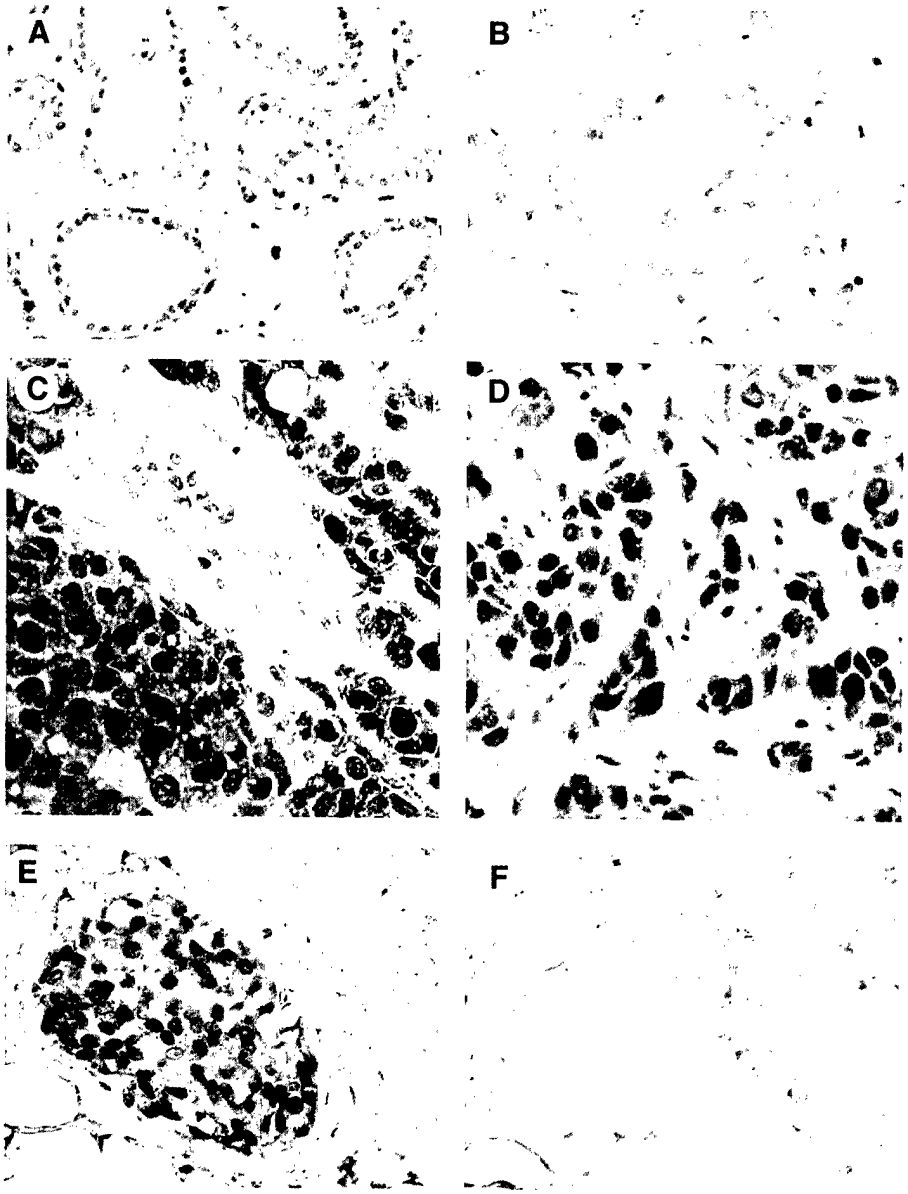


Fig. 1. Immunostaining of Pin1 in human breast cancer. Sections from paraffin-embedded tissues were subjected to an antigen retrieval treatment, followed by immunostaining with anti-Pin1 antibodies. Non-cancerous tissues (**A** and **B**; normal breast with mild fibrocystic changes) show weak, but detectable, Pin1 staining, while invasive ductal carcinomas (**C** and **D**) or ductal carcinoma *in situ* (**E**) show intense Pin1 staining. To show the specificity of Pin1 antibodies, Pin1-specific antibodies were first depleted using GST–Pin1 beads and then used to stain the breast sections (**F**).

ation (Yaffe *et al.*, 1997; Shen *et al.*, 1998; Lu *et al.*, 1999b; Zhou *et al.*, 1999, 2000). For example, in the case of Cdc25C, Pin1 binds phosphorylated Cdc25C, and inhibits its activity to dephosphorylate and activate Cdc2 (Shen *et al.*, 1998; Zhou *et al.*, 2000). However, in the case of tau, Pin1 binds Alzheimer's disease-associated phosphorylated tau and restores its biological function to promote microtubule assembly (Lu *et al.*, 1999a; Zhou *et al.*, 2000). These results indicate that Pin1 plays an important role in the regulation of a defined subset of phosphorylated proteins.

Functionally, Pin1 is critical for cell proliferation *in vivo*. Temperature-sensitive mutations or deletion of the Ess1 gene (the Pin1 homologue in budding yeast) result in mitotic arrest and nuclear fragmentation (Hanes *et al.*, 1989; Hani *et al.*, 1995, 1999; Lu *et al.*, 1996). These arrested cells have defective 3′ end formation of pre-mRNA, and decreased levels of some mRNAs (Hani *et al.*, 1999; Wu *et al.*, 2000). However, it remains to be determined whether these defects are primarily due to the effect of Ess1 on the general transcription machinery, as suggested, or secondarily due to the fact that these cells are arrested in mitosis with fragmented nuclei, or both. Inhibition of the Pin1 function in human tumor cells using expression of the Pin1 antisense RNA or dominant-negative mutants induces mitotic arrest and apoptosis (Lu *et al.*, 1996; Rippmann *et al.*, 2000; P.J.Lu, X.Z.Zhou, Y.-C.Liou, J.P.Noel and K.P.Lu, submitted). Similarly, depletion of Pin1 in Alzheimer's disease brain may also contribute to neuronal death (Lu *et al.*, 1999a). Furthermore, depletion of Pin1 in *Xenopus* extracts induces premature mitotic entry and disrupts a DNA

replication checkpoint (Winkler *et al.*, 2000). These results together suggest that the level and function of Pin1 are pivotal for cell proliferation. However, the level and role of Pin1 in human cancer have not yet been described.

Here we show that Pin1 is overexpressed in most human breast cancer cell lines and many human breast cancer tissues. Furthermore, the Pin1 levels correlate significantly with the grade of the tumors, according to Bloom and Richardson's classification system (Bloom and Richardson, 1957), and with the level of cyclin D1 in the tumors. Moreover, Pin1 increases levels of cellular cyclin D1 mRNA and protein, and activates its promoter through the AP-1 site. Importantly, Pin1 binds to phosphorylated c-Jun and increases its transcriptional activity towards the cyclin D1 promoter, in cooperation either with activated JNK or oncogenic Ras. The effects of Pin1 on the c-Jun transcriptional activity depend on both the isomerase activity of Pin1 and phosphorylation of c-Jun on $Ser^{63/73}$. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun. These results demonstrate that Pin1 is up-regulated in human tumor samples and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. Given the crucial roles of the activated Ras signaling and cyclin D1 overexpression in the development of cancer, our results suggest that overexpression of Pin1 may promote tumor growth.

Results

Pin1 is overexpressed in human breast tumors and its levels correlate with the tumor grade

To examine the role of Pin1 in cancer, we examined the expression of Pin1 in normal human breast tissues and breast tumors by immunohistochemistry and immunoblotting with affinity-purified anti-Pin1 antibodies, as described earlier (Lu *et al.*, 1999a). Normal breast epithelial cells showed weak but detectable Pin1 staining primarily in the nucleus (Figure 1A and B). In contrast, carcinoma cells were strongly positive for the Pin1 staining (Figure 1C–E), while surrounding normal connective tissue, blood vessels, adipose and stromal cells stained only weakly for Pin1 (Figure 1E). In these tumor cells, Pin1 staining was detected at high levels in the cytoplasm, in addition to intensive staining in the nucleus (Figure 1C–E). To ensure that these signals indeed represent Pin1, the Pin1-specific antibodies were depleted using glutathione *S*-transferase (GST)–Pin1 beads prior to immunostaining. Figure 1F shows that the Pin1-depleted antibodies showed no immunoreactivity, confirming the specificity of the antibodies, as described (Lu *et al.*, 1999a). Immunohistochemistry in other cancer types revealed high Pin1 levels in some tumors, including colon cancer, lymphomas, melanoma, prostate and brain tumors, but rarely in others, such as sarcoma (data not shown). Since we had access to a large number of breast cancer samples, we focused this study on breast cancer.

To evaluate Pin1 expression in breast cancer quantitatively, we ground fresh, normal or tumor breast tissues in liquid nitrogen and subjected the lysates directly to immunoblotting analysis with various antibodies, followed by semi-quantification of protein levels using Imagequant, as described (Lu *et al.*, 1999a). Pin1 was generally detected as a doublet in immunoblots, especially in tumor tissues where Pin1 was overexpressed. Upon dephosphorylation with protein phosphatases PP2A and PP1, or calf intestine phosphatase (CIP), the intensity of the upper band decreased, while the lower one increased (Figure 2B). In addition, Pin1 displays a mitosis- and phosphorylation-specific mobility shift during the cell cycle (P.J.Lu, X.Z.Zhou, Y.-C.Liou, J.P.Noel and K.P.Lu, submitted). These results indicate that the Pin1 doublet is likely to be due to the electrophoretic mobility difference of phosphorylated and dephosphorylated Pin1. Interestingly, the upper phosphorylated band appeared to be predominant in the normal tissues, whereas the lower dephosphorylated band was more abundant in the cancerous tissues where Pin1 was overexpressed (Figure 2A), suggesting that there are more mitotic cells and/or the kinase(s) responsible for the Pin1 phosphorylation might be limited in tumor cells.

To compare the levels of Pin1 in different human tissues, we used actin as an internal control, and expressed the Pin1 level in each sample as a Pin1:actin ratio. We defined Pin1 overexpression as higher than the mean plus three times the standard deviation ($\bar{x}$ + 3 SD) of normal controls (Figure 2C; Table I). In 10 normal and 51 primary human breast cancer tissues examined, we observed striking differences in the levels of Pin1 protein expression (Figure 2A and C). One out of four DCIS tumors, 20 out of 28 (71.4%) grade II tumors and 17 out of 19 (89.5%) grade III tumors, according to Bloom and Richardson's classification system, overexpressed Pin1 (Figure 2C). Although we observed considerable inter-individual variations, especially in grade II and III tumors (Figure 2C), the mean expression level of Pin1 was ~10 times higher in cancer samples than in the normal controls (Table I). Furthermore, Pin1 levels positively correlated with the Bloom and Richardson grade in invasive breast cancer, as analyzed by the Kruskal–Wallis test (Glantz, 1997) (Figure 2B; Table II). Similar results were also obtained using a monoclonal antibody against Pin1 for immunostaining and immunoblotting analyses (data not shown). The levels of Pin1 in four cell lines derived from human breast cancers were considerably higher than those in either normal mammary epithelial cells or two cell lines established from normal mammary epithelial cells (Figure 2D). Together, these results indicate that Pin1 is overexpressed in many human breast cancer tissues and cell lines, and its levels are correlated with the tumor grade.

Up-regulation of Pin1 correlates with cyclin D1 levels in breast tumor tissues and elevates cellular cyclin D1 expression in breast cell lines

Amongst other breast cancer tumor markers, Pin1 levels did not appear to correlate with either estrogen receptor or HER2/neu expression, but did correlate significantly with cyclin D1 overexpression (Tables I and II). As shown previously (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), cyclin D1 was overexpressed in ~50% of the patient samples (24 out of 51 cases). Importantly, Pin1 was overexpressed in 20 out of 24 cyclin D1-overexpressing tumors, and Pin1 levels in cyclin D1-overexpressing

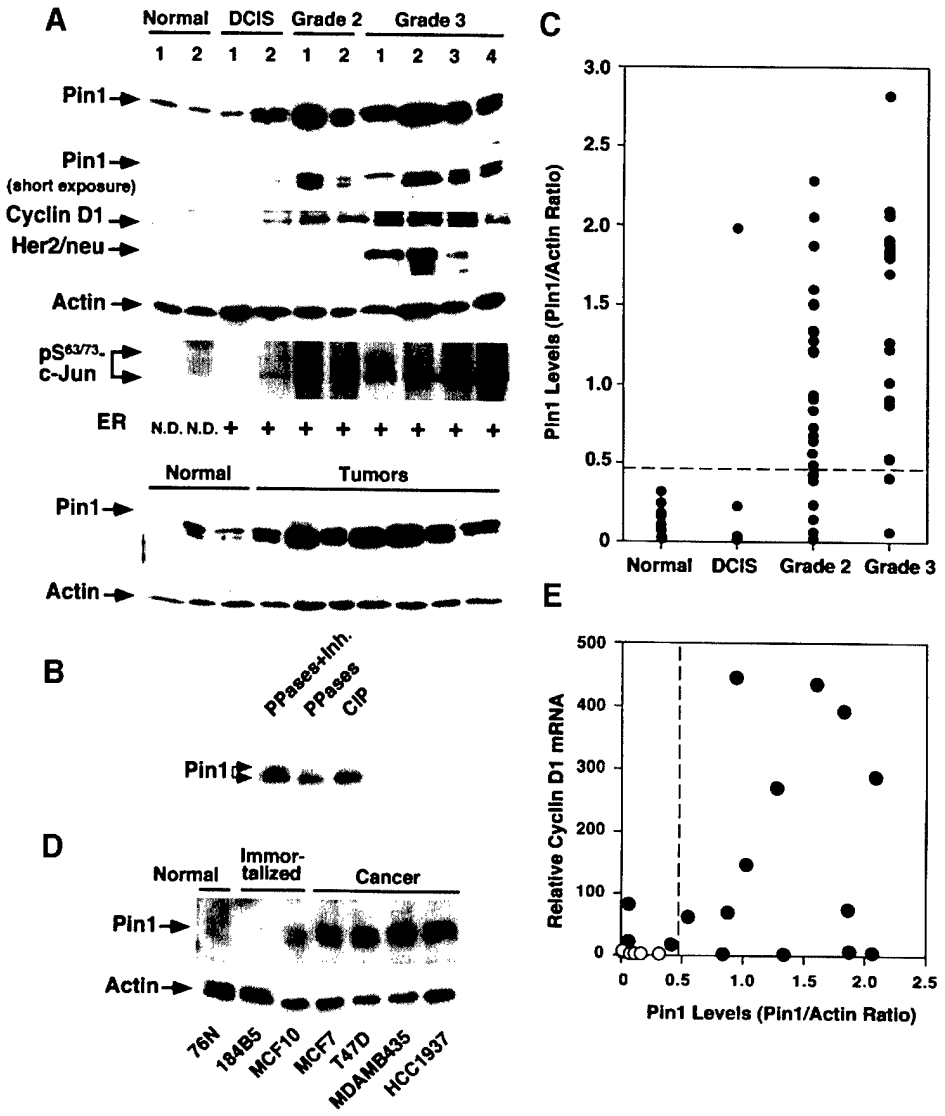


Fig. 2. Pin1 overexpression in human breast cancer cell lines and patient tissues, and its correlation with the Bloom and Richardson grade of tumors. (**A**) Comparison of Pin1 levels and known breast tumor markers in normal and cancerous human breast tissues. Normal breast and cancer tissues were pulverized in liquid nitrogen, and equal amounts of total protein were separated on SDS-containing gels and transferred to membranes. The membranes were cut into five pieces and subjected to immunoblotting analysis using antibodies to Pin1, cyclin D1, HER2/neu, phosphorylated $Ser^{63/73}$-c-Jun and actin, respectively. The estrogen receptor status was determined by radioimmunoassay and defined as positive when its levels were >10 fmol/l. The estrogen receptor status in normal controls was not determined (N.D.). Note that Pin1 was detected in immunoblots as a doublet due to phosphorylation. (**B**) Phosphatase treatment abolishes the double-band pattern of Pin1 in immunoblots. Tumor cell lysates were treated either with a mixture of PP1 and PP2A (PPases) in the presence (lane 1) or absence (lane 2) of the phosphatase inhibitor okadaic acid (Inh.), or CIP (lane 3). (**C**) Pin1 levels in 10 normal breast tissues and different stages of 51 human breast cancer samples. Pin1 levels were determined by immunoblotting analysis, as in (A), and semi-quantified using Imagequant. Actin was used as an internal control, and the Pin1 level in each sample was expressed as the Pin1:actin ratio. (**D**) Comparison of Pin1 levels in mammary epithelial cell lines. The same amounts of total lysates prepared from normal human mammary epithelial cell lines (Normal), spontaneously immortalized normal human mammary epithelial cell lines (Immortalized) and human breast carcinoma-derived cell lines (Cancer) were subjected to immunoblotting analysis with Pin1 or actin antibodies. (**E**) Correlation of Pin1 protein levels with cyclin D1 mRNA. RNA was isolated from six normal and 16 cancerous tissues, cDNA synthesized and subjected to real-time PCR for the quantitative analysis of cyclin D1 mRNA expression. The Pearson correlation coefficient was 0.47 (p <0.05).

tumors were on average about twice as high as those in cyclin D1-negative tumors (Figure 2A; Table II). In order to establish a link between Pin1 overexpression and cyclin D1 transcription, we performed quantitative real-time PCR to detect cyclin D1 mRNA expression in 6 out of the 10 normal tissues and 16 out of the 51 breast cancer tissues, from which we were able to isolate total RNA. Figure 2E shows relative cyclin D1 mRNA levels as a function of Pin1 protein levels. While a few patients had high Pin1 but low cyclin D1 mRNA levels, all but one patient with high cyclin D1 mRNA levels also displayed high Pin1 levels, which is consistent with the results on cyclin D1 protein levels (Table II). Statistical analysis revealed that there was again a positive correlation between Pin1 protein levels and cyclin D1 mRNA expression (r = 0.47, p <0.05).

The correlation between Pin1 and cyclin D1 expression suggested that overexpression of Pin1 might increase the

Table I. Clinical and pathological characteristics of breast tissues

	Normal	Carcinoma			
		Total	*In situ*	Grade 2	Grade 3
Pin1 positive	0/10[a]	38/51 (75%)	1/4 (25%)	20/28 (71%)	17/19 (89%)
$\bar{x} \pm$ SD	0.114 ± 0.106	1.072 ± 0.719	0.564 ± 0.948	0.924 ± 0.609	1.399 ± 0.717
Cyclin D1	0/10	24/51 (47%)	2/4 (50%)	10/28 (36%)	12/19 (63%)
HER2/neu	0/10	8/51 (16%)	0/4 (0%)	4/28 (14%)	4/19 (21%)
Estrogen receptor	N.D.[b]	34/50[c] (68%)	3/4 (75%)	20/28 (71%)	11/18 (61%)
Age median (range)	57 (22–91)	65 (28–90)	72 (43–80)	65 (31–90)	60 (28–78)

Tumors were pathologically classified into ductal carcinoma *in situ* (*in situ*) and invasive grade 2 and 3 carcinoma, according to the criteria of Bloom and Richardson. Levels of Pin1 in tissues were determined by immunoblotting analysis and semi-quantified using Imagequant, with the results being expressed as Pin1/actin ratio. Pin1 was defined positive when the Pin1/actin ratio was higher than the mean plus three times the standard deviation ($\bar{x}$ ± 3 SD) of normal controls. Cyclin D1 and HER2/neu were determined by immunoblotting and categorized as either positive or negative by the presence or absence of the respective proteins. Estrogen receptor was defined positive when its levels were >10 fmol/l, as determined by radioimmunoassay.

[a]Number of cases examined.

[b]Estrogen receptors in controls not determined.

[c]Estrogen receptor determination on one patient not available.

Table II. Correlation of the Pin1 level with clinical and pathological characteristics

	No. of cases	Pin1 level ($\bar{x}$ ± SD)	*p*
Normal	10	0.114 ± 0.106	<0.0001[b]
Tumor	51	1.072 ± 0.716	
Tumor grade			
grade 2	28	0.924 ± 0.609	0.02[b]
grade 3	19	1.399 ± 0.717	
Cyclin D1[a]			
positive	24	1.364 ± 0.715	0.01[b]
negative	27	0.824 ± 0.631	
HER2/neu[a]			
positive	8	1.317 ± 0.732	0.10
negative	43	1.027 ± 0.713	
Estrogen receptor[a]			
positive	34	1.011 ± 0.718	0.32
negative	16	1.238 ± 0.720	

The significance of the differences in Pin1 levels between various clinical and pathological categories was analyzed by the Kruskal–Wallis test.

[a]Analyses performed only on tumors.

[b]The differences are statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$.

expression of endogenous cyclin D1. To examine this possibility, we transiently transfected a Pin1 expression construct into two breast cancer-derived cell lines, MCF7 and T47D cells, and then examined the effects on endogenous cyclin D1 levels. Pin1 overexpression led to 2- to 3-fold increases in cyclin D1 protein levels in both cell lines, while the expression of actin remained constant (Figure 3A). To examine whether the depletion of Pin1 affected cyclin D1 expression, we used MCF7 and HeLa cells because their Pin1 levels can be increased or decreased by expressing a sense or antisense Pin1 construct, respectively (Figure 3B), as described previously (Lu *et al.*, 1996). Overexpression of Pin1 significantly increased the levels of cyclin D1 protein and mRNA in both cells (Figure 3B and C and data not shown). In contrast, depletion of Pin1 significantly reduced the levels of cyclin D1 protein and mRNA in MCF7 cells (Figure 3B and C). Since these experiments were performed between 24 and 36 h after transfection, and since manipulation of Pin1 levels affects the cell cycle only after 48–72 h post-transfection (Lu *et al.*, 1996), the observed effects of Pin1 on cyclin D1 are unlikely to be related to cell cycle arrest. These results indicate that high levels of Pin1 correlate with the overexpression of cyclin D1 on both RNA and protein levels in human breast cancer tissues, and that overexpression of Pin1 increases cellular cyclin D1 mRNA and protein levels in cell lines.

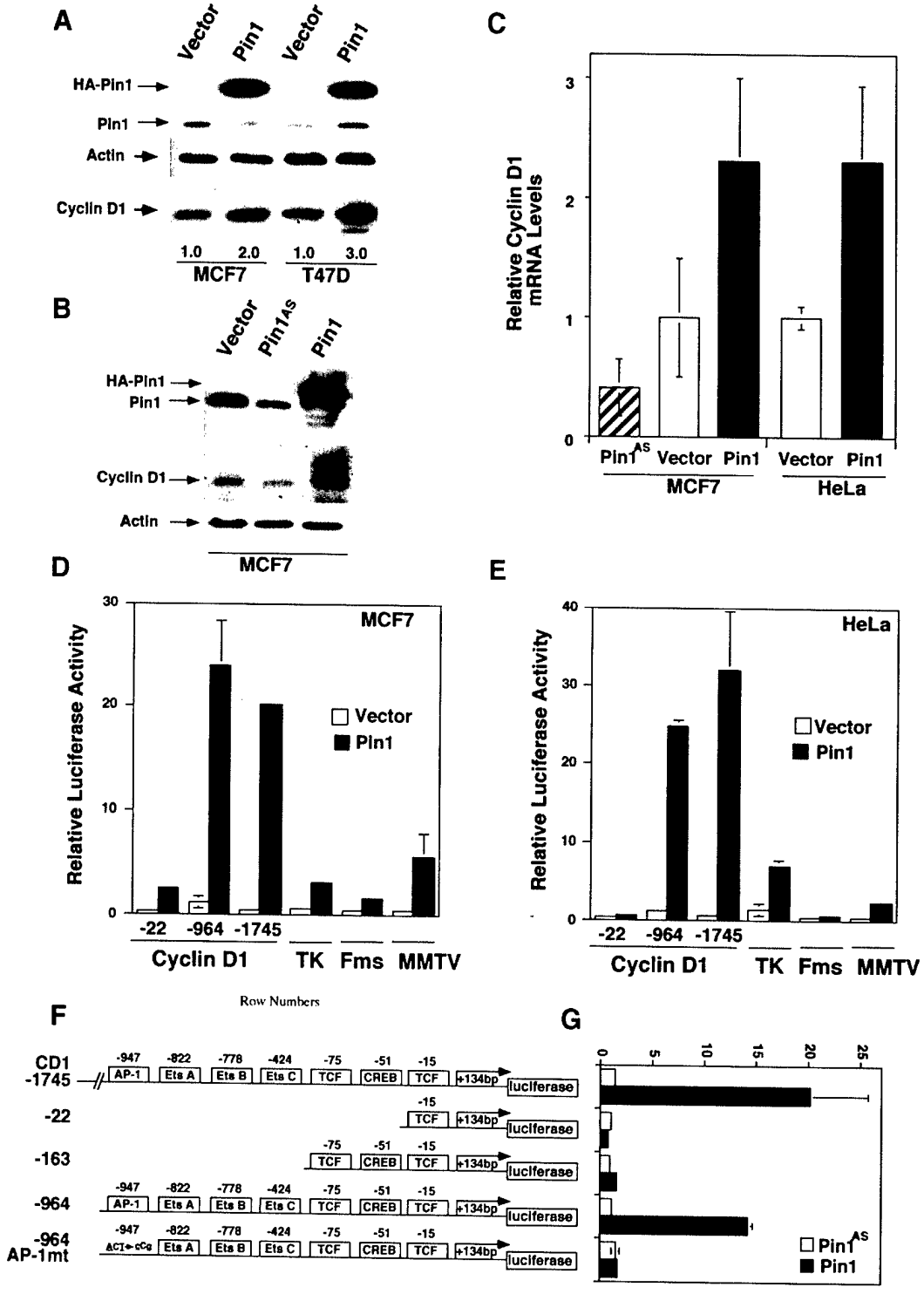
Pin1 activates the cyclin D1 promoter

Although cyclin D1 overexpression is found in ~50% of breast cancer patients (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), gene amplification accounts for only 10% of these cases (Fantl *et al.*, 1993). Therefore, other mechanisms, such as up-regulation of gene transcription, must play a substantial role in the overexpression of cyclin D1. To determine whether Pin1 regulates the transcription of cyclin D1, we measured the effects of Pin1 on the cyclin D1 promoter using cyclin D1–luciferase reporter constructs. Two cyclin D1–reporter constructs were tested: one (–1745CD1) corresponds to the original fragment of cyclin D1 5′ sequence cloned from the PRAD1 breakpoint (Motokura and Arnold, 1993), and the other (–964CD1) is the minimum 5′ sequence that retains the responsiveness to activated Ras (Albanese *et al.*, 1995). Both –1745CD1 and –964CD1 reporters were strongly activated in response to expression of Pin1 both in MCF7 and HeLa cells (Figure 3D and E). These results indicate that Pin1 activates the cyclin D1 promoter and that the –964CD1 promoter fragment retains the complete responsiveness to Pin1.

It has recently been shown that Pin1/Ess1p binds the phosphorylated C-terminal domain of RNA polymerase II and may regulate the general transcription machinery in yeast (Wu *et al.*, 2000). To determine whether activation of the cyclin D1 promoter by Pin1 is due to its effect on the general transcription machinery, we examined the effect of Pin1 on several other unrelated promoters. To detect the maximal effect of Pin1 on various promoters, we used 500 ng of Pin1 cDNA per transfection. In contrast, out of many other promoters examined, including thymidine

kinase (TK), c-fms (M-CSF receptor) and MMTV promoters, Pin1 either had no effect or had minor transactivating effects (Figure 3D and E), indicating that activation of the general transcription machinery by Pin1 is very low, which is consistent with a recent report (Chao *et al.*, 2001). Therefore, the above results indicate that Pin1 specifically activates the cyclin D1 promoter.

To further confirm the specificity of the Pin1 action on the cyclin D1 promoter, we identified the element in the cyclin D1 promoter that is responsible for Pin1 activation. The –964CD1 promoter fragment contains binding sites for various transcription factors, including a CREB site, four TCF sites, three Ets sites and one AP-1 site (Albanese *et al.*, 1995; Tetsu and McCormick, 1999) (Figure 3F). To determine which promoter is necessary for Pin1 responsiveness, we used two deletion constructs containing either 22 bp (–22CD1) or 163 bp (–163CD1) of the cyclin D1 promoter as reporters. Low concentrations (50–200 ng) of Pin1 did not have any significant transactivating effect either on the –22CD1 or the –163CD1 reporter (Figure 3F



and G), while at high concentrations (>200 ng per transfection) Pin1 could also transactivate the –163CD1 promoter containing the TCF sites (Ryo *et al.*, 2001). At low concentrations, i.e. ≤200 ng, Pin1 significantly transactivated both the –1745CD1 and –964CD1 promoters (Figure 3F and G). These results confirm that Pin1 does not affect the cyclin D1 promoter activity via the general transcriptional machinery but through specific sequences such as the AP-1 and/or Ets sites. To examine the importance of the AP-1 site, we used a mutant promoter, –964CD1AP-1mt, containing only two base-pair substitutions at the consensus AP-1 site, as described (Albanese *et al.*, 1995). Elimination of the AP-1 site almost completely abolished the ability of Pin1 to activate the cyclin D1 promoter (Figure 3F and G). These results indicate that the AP-1 site is essential for activation of the cyclin D1 promoter by Pin1.

Pin1 binds c-Jun phosphorylated on Ser63/73-Pro motifs

The AP-1 site mutation in the cyclin D1 promoter that disrupts the Pin1 transactivating activity also abolishes cyclin D1 expression induced by the activation of Ras or c-Jun (Albanese *et al.*, 1995), suggesting that Pin1 might affect the same pathway as that regulated by Ras or c-Jun. Activation of Ras triggers a signaling cascade, leading to activation of the c-Jun N-terminal kinase JNK, which phosphorylates c-Jun on $Ser^{63/73}$-Pro to increase its transcriptional activity towards its target genes, including cyclin D1 (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Derijard *et al.*, 1994; Hinds *et al.*, 1994; Albanese *et al.*, 1995, 1999; Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Bakiri *et al.*, 2000). In fact, Ras-mediated tumorigenesis depends on signaling pathways with cyclin D1 as an important intermediary protein (Robles *et al.*, 1998). Since Pin1 binds and regulates the function of a defined subset of proteins phosphorylated on certain Ser/Thr-Pro motifs (Shen *et al.*, 1998; Lu *et al.*, 1999a), it is possible that Pin1 might activate the cyclin D1 promoter via modulation of the the activity of phosphorylated c-Jun.

A well established and successful procedure to identify Pin1 substrates has been the use of GST–Pin1 pulldown experiments to determine whether Pin1 binds to c-Jun, and whether the binding depends on phosphorylation of c-Jun on specific Ser-Pro motifs, as demonstrated for many other Pin1 substrates (Yaffe *et al.*, 1997; Crenshaw *et al.*, 1998; Shen *et al.*, 1998; Lu *et al.*, 1999b). To increase phosphorylation of c-Jun on $Ser^{63/73}$-Pro, we co-transfected c-Jun with a constitutively activated form of JNK (Derijard *et al.*, 1994). Alternatively, we co-transfected c-Jun with a further upstream activator, the oncogenic Harvey-Ras (Ha-Ras or RasL61), which activates a MAK kinase pathway, leading to activation of JNK (Smeal *et al.*, 1991; Derijard *et al.*, 1994). To reduce phosphorylation of c-Jun on $Ser^{63/73}$-Pro, we co-transfected c-Jun with the dominant-negative Ras (DN-Ras or RasN17) (Smeal *et al.*, 1991; Derijard *et al.*, 1994). As expected, phosphorylation of c-Jun on $Ser^{63/73}$-Pro was increased to similar extents by either activated JNK or Ha-Ras, but significantly decreased by DN-Ras, as detected by antibodies specifically recognizing phosphorylated $Ser^{63/73}$ in c-Jun (Figure 4A and B). Notably, following activation of JNKs by UV radiation or serum stimulation, c-Jun has been shown to be phosphorylated on several Ser-Thr sites, which resulted in a considerable shift in electrophoretic mobility of the protein, migrating as multiple bands in SDS gels (Ui *et al.*, 1998). Furthermore, mutation of c-Jun on Ser63 and Ser73 abolishes the mobility shift (Ui *et al.*, 1998). We observed a similar mobility shift for wild-type c-Jun, but not $c\text{-}Jun^{S63/73A}$, after co-transfection either with Ha-Ras or activated JNK (Figure 4A–D). Importantly, although there was no binding between GST and c-Jun, weak binding between GST–Pin1 and c-Jun was detected when only c-Jun was transfected (Figure 4C). Furthermore, the binding was significantly increased by co-transfection either with activated JNK or oncogenic Ha-Ras, but not with DN-Ras (Figure 4C). Moreover, c-Jun bound by Pin1 was also phosphorylated on $Ser^{63/73}$-Pro, as indicated by phosphorylated $Ser^{63/73}$-specific antibodies (Figure 4D). To examine further the importance of phosphorylation on $Ser^{63/73}$ for Pin1 binding, we used a c-Jun mutant, $c\text{-}Jun^{S63/73A}$, which contains double Ala substitutions at Ser63 and Ser73 (Smeal *et al.*, 1991). In contrast to wild-type c-Jun, the mutant protein did not display a significant mobility shift and was not recognized by phosphorylated $Ser^{63/73}$-specific antibodies (Figure 4A and B), as shown previously (Ui *et al.*, 1998).

Fig. 3. Pin1 elevates cyclin D1 protein and activates the cyclin D1 promoter via the AP-1 site. (**A**) Increase in cellular cyclin D1 protein by Pin1. MCF7 or T47D cells were transfected with Pin1 or control vector, followed by immunoblotting analysis of the cell lysates with antibodies against Pin1 and cyclin D1, with actin as a control. Cyclin D1 levels were semi-quantified using Imagequant and are presented below the image; the level in the vector control was defined as 1. (**B**) Manipulation of Pin1 levels in cells causes changes in cyclin D1 levels. MCF7 cells were transiently transfected with the control vector or a construct expressing HA-Pin1 or antisense Pin1 ($Pin1^{AS}$), followed by immunoblotting analysis with anti-cyclin D1, -Pin1 or -actin antibodies. (**C**) Overexpression and depletion of Pin1 increase and decrease levels of cyclin D1 mRNA. MCF7 or HeLa cells were transfected with constructs encoding for Pin1 sense, antisense or vector control as indicated in the figure. After 24 h, mRNA was isolated, cDNA synthesized and subjected to real-time PCR to obtain relative cyclin D1 mRNA levels. (**D** and **E**) Activation of cyclin D1, but not TK, c-fms or MMTV promoter by Pin1. MCF7 (D) or HeLa (E) cells were transiently transfected with Pin1 or the vector and various reporter constructs, followed by assaying the luciferase activity. pRL-TK *Renilla* luciferase reporter construct was co-transfected in each sample to normalize for transfection efficiency. The activity of the reporter luciferase was expressed relative to that in control vector-transfected cells, which is defined as 1. All results are expressed as $\bar{x} \pm$ SD of independent duplicate cultures. Note that to detect the maximal effect of Pin1 on various promoters, we used 0.5 μg of Pin1 cDNA per transfection in this experiment, which was higher than in other experiments described here. (**F**) Schematic representation of cyclin D1 (CD1) pA3LUC basic reporter constructs and its mutants. Possible transcription factor-binding sites are indicated. –964CD1AP-1mt was same as the wild-type –964CD1construct except for two base-pair substitutions at the consensus AP-1 site. (**G**) Activation of the cyclin D1 promoter by Pin1 via the AP-1 site. HeLa cells were co-transfected with various cyclin D1 reporter constructs as indicated in (F) and Pin1 sense or antisense ($Pin1^{AS}$) construct, followed by assaying the luciferase activity. Note that for this experiment 200 ng Pin1 sense or antisense cDNA were used, while in subsequent co-transfection experiments only 50 ng/assay were used.

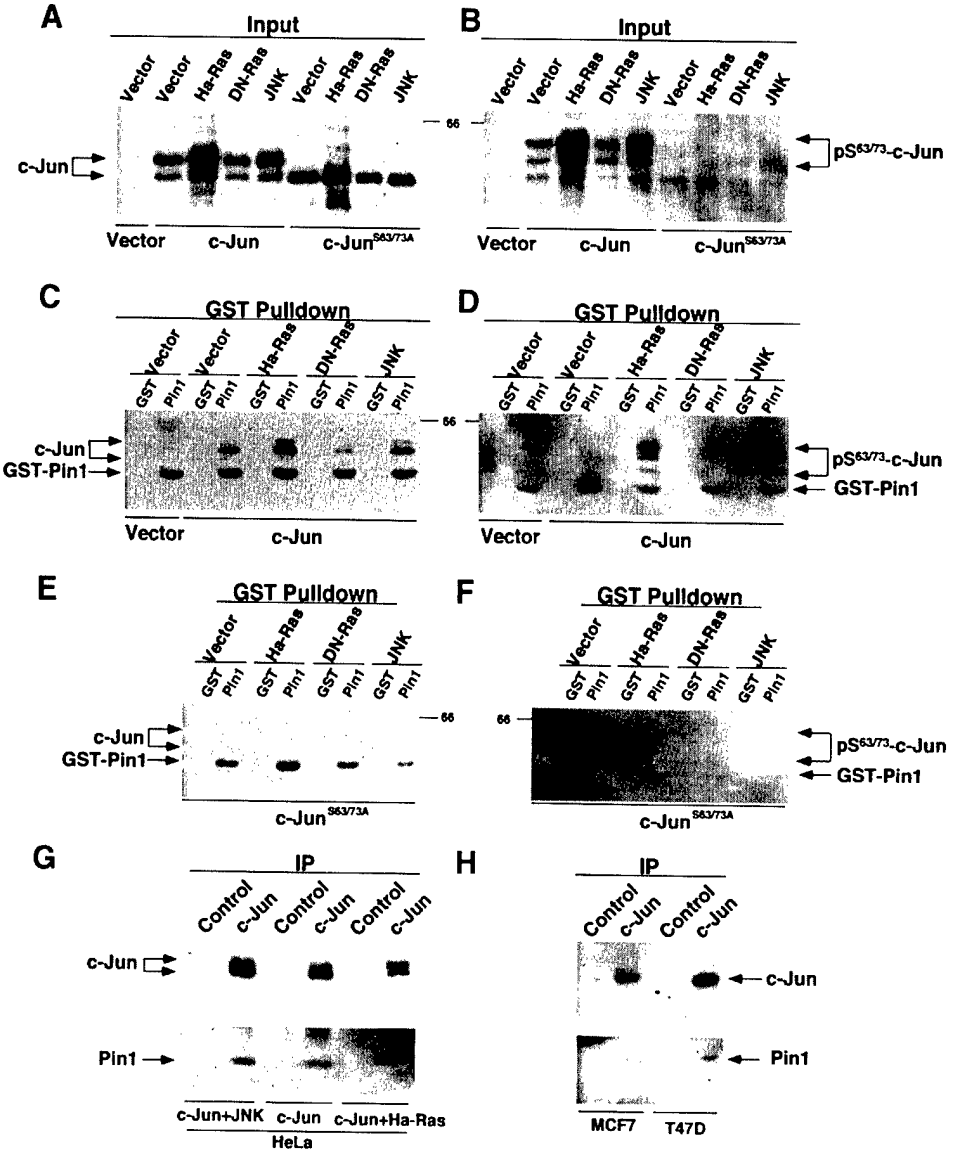


Fig. 4. Pin1 binds to c-Jun phosphorylated on Ser$^{63/73}$-Pro. (**A** and **B**) Modulation of c-Jun phosphorylation by Ras or JNK. HeLa cells were co-transfected with c-Jun or c-Jun$^{S63/73A}$ and Ha-Ras, DN-Ras, activated JNK or control vector. Cells were harvested and cellular proteins were subjected to immunoblotting analysis with antibodies against c-Jun (A) or phosphorylated Ser$^{63/73}$-c-Jun (B). (**C** and **D**) Interaction between Pin1 and c-Jun phosphorylated on Ser$^{63/73}$-Pro. The same cellular proteins as those described in (A) were incubated with GST–agarose beads that had been pre-incubated with either GST alone or GST–Pin1. Proteins associated with the beads were subjected to immunoblotting analysis with antibodies against c-Jun (C) or phosphorylated Ser$^{63/73}$-c-Jun (D). Note that GST–Pin1 was non-specifically recognized by monoclonal antibodies, as shown previously (Yaffe *et al.*, 1997; Lu *et al.*, 1999b). (**E** and **F**) No interaction between Pin1 and c-Jun$^{S63/73A}$. The same cellular proteins as those described in the (A) were incubated with GST–agarose beads containing GST or GST–Pin1, and bound proteins were subjected to immunoblotting analysis with antibodies against c-Jun (E) or phosphorylated Ser$^{63/73}$-c-Jun (F). (**G** and **H**) Co-immunoprecipitation of transfected (G) or endogenous (H) c-Jun with endogenous Pin1. HeLa cells were co-transfected with c-Jun and Ha-Ras or JNK. c-Jun was immunoprecipitated from transfected HeLa cells (G) or non-transfected breast cancer cell lines (H) with polyclonal c-Jun antibodies or non-related antibodies (Control), and then subjected to immunoblotting using monoclonal anti-c-Jun antibodies (upper panel) or anti-Pin1 antibodies (lower panel).

Importantly, little, if any, mutant protein was precipitated by Pin1 (Figure 4E and F). These results indicate that phosphorylation of c-Jun on Ser$^{63/73}$-Pro is important for the Pin1 binding. Thus, Pin1 binds to c-Jun via phosphorylated Ser$^{63/73}$-Pro motifs.

To confirm these GST–Pin1 protein pulldown results, we performed co-immunoprecipitation experiments between endogenous Pin1 and transfected c-Jun in the presence or absence of activated JNK or Ha-Ras, as well as co-immunoprecipitations between endogenous Pin1 and c-Jun in breast cancer cell lines expressing high levels of both proteins. Endogenous Pin1 was detected in anti-c-Jun immunoprecipitates from transfected (Figure 4G) and non-transfected cells (Figure 4H). Furthermore, more Pin1 was co-immunoprecipitated by anti-c-Jun antibodies if c-Jun was co-transfected with activated JNK or Ha-Ras (Figure 4G). These results indicate that Pin1 binds c-Jun *in vivo* in breast cancer cell lines, and that the binding is increased when c-Jun is phosphorylated on Ser$^{63/73}$-Pro motifs by activated JNK or Ha-Ras. These results demonstrate that Pin1 binds phosphorylated c-Jun both *in vitro* and *in vivo*.

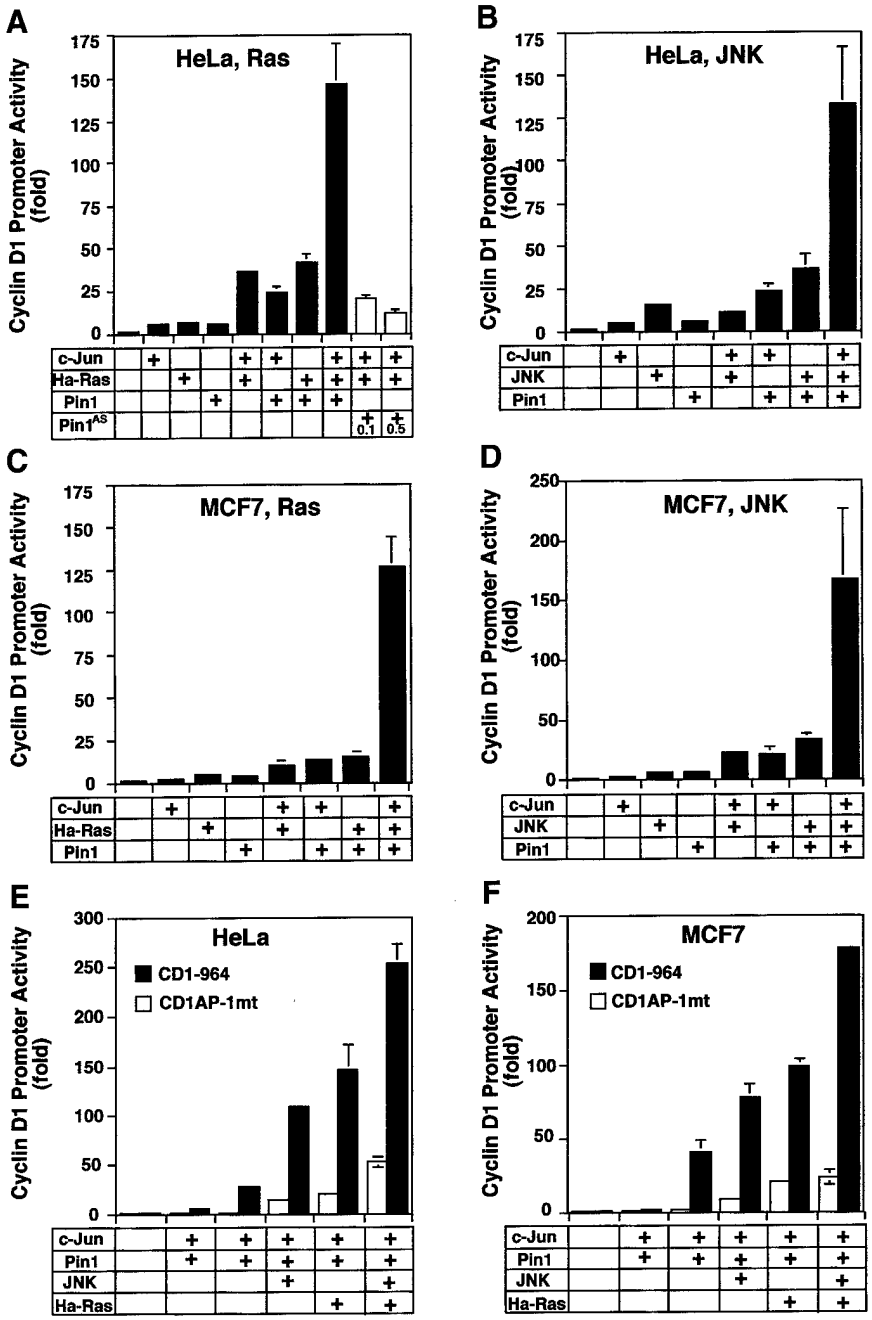


Fig. 5. Pin1 cooperates either with Ha-Ras or activated JNK in enhancing the activity of c-Jun to activate the cyclin D1 promoter. HeLa cells (**A**, **B** and **E**) or MCF7 (**C**, **D** and **F**) were co-transfected with vector, c-Jun, or c-Jun with or without Ha-Ras (A and C) or activated JNK (B and D) and then subjected to the luciferase assay with the –964 cyclin D1 Luc promoter construct as reporter gene. In the same system, a reporter gene construct with an AP-1 site mutant fails to respond to Pin1 in combination with c-Jun, JNK or Ha-Ras (E and F).

Pin1 cooperates with either oncogenic Ha-Ras or activated JNK to increase transcriptional activity of c-Jun towards the cyclin D1 promoter

Given that Pin1 binds phosphorylated c-Jun, we asked whether Pin1 also modulates the activity of c-Jun. To address this question, we examined the effect of Pin1 on the transcriptional activity of c-Jun towards the cyclin D1 promoter in the presence or absence of Ha-Ras or activated JNK. When Pin1 cDNA was co-transfected with c-Jun, Pin1 cooperated moderately with c-Jun in activating the cyclin D1 promoter in both MCF7 and HeLa cells (Figure 5). The activity of the cyclin D1 promoter in cells co-transfected with Pin1 and c-Jun was 3- to 5-fold higher than that in cells transfected with either Pin1 or c-Jun alone (Figure 5A–D). The most dramatic potentiation of cyclin D1 reporter gene activity was observed when c-Jun was activated by JNK or Ha-Ras in the presence of Pin1; cyclin D1 promoter activity was increased up to 150-fold, or higher, in both cell lines (Figure 5A–D). The combination of JNK, Ras, c-Jun and Pin1 resulted in a further small increase in transactivation (Figure 5E and F, last bars), consistent with the idea that Ras and JNK act on

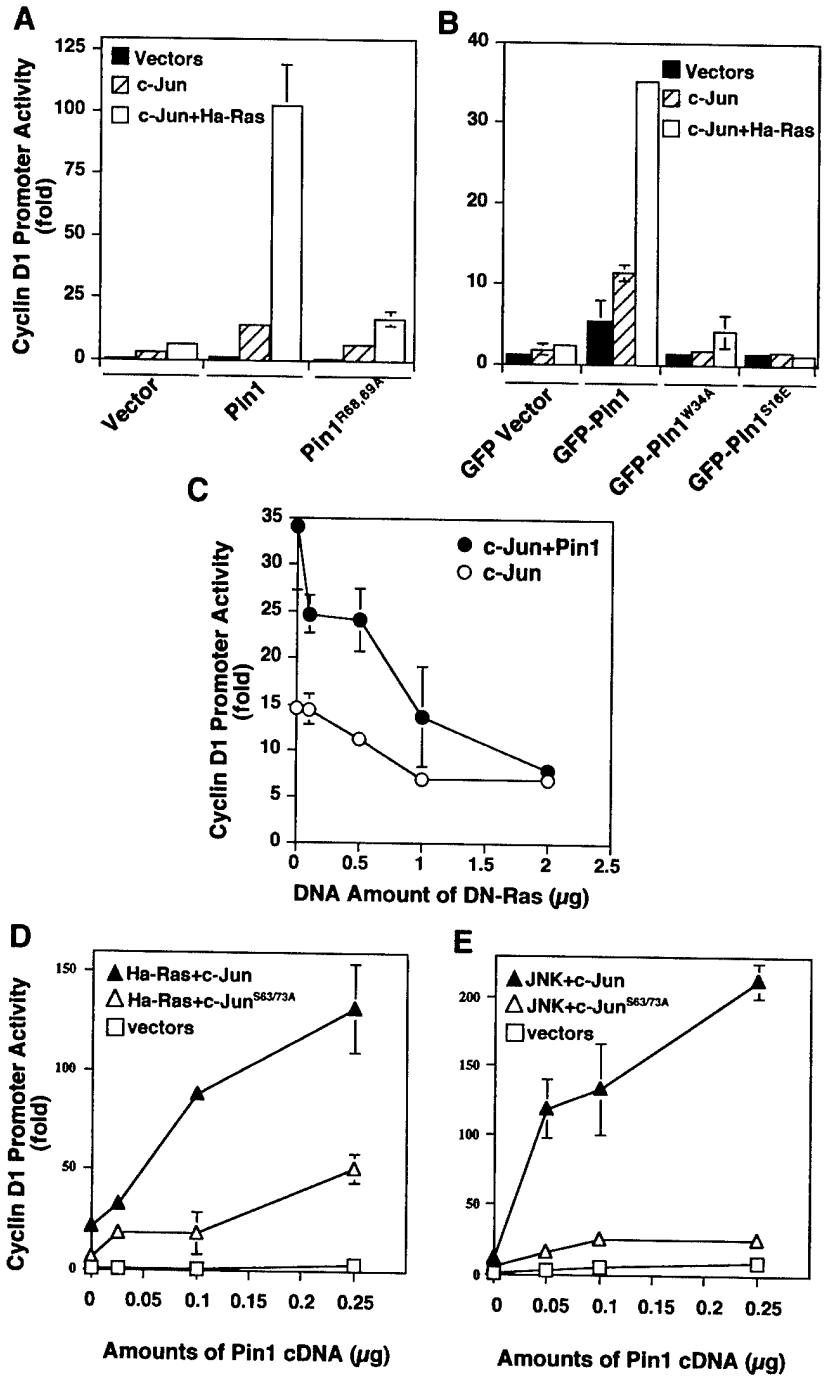


Fig. 6. The effects on the transcriptional activity of c-Jun depend on the phosphoprotein-binding and PPIase activity of Pin1 as well as phosphorylation of c-Jun on $Ser^{63/73}$. (**A**) Abolishing the Pin1 effect by inactivating its PPIase activity. HeLa cells were co-transfected with vectors, c-Jun, or c-Jun + Ha-Ras, and Pin1 or its PPIase-negative mutant $Pin1^{R68,69A}$, and then subjected to luciferase assay. $Pin1^{R68,69A}$ fails to isomerize phosphorylated Ser/Thr-Pro bonds (Yaffe *et al.*, 1997). (**B**) Abolishing the Pin1 effect by inactivating its phosphoprotein-binding activity. HeLa cells were co-transfected with vectors, c-Jun, or c-Jun + Ha-Ras and green fluorescent protein (GFP)–Pin1 or its WW domain point mutants, and then subjected to luciferase assay. GFP–$Pin1^{W34A}$ and GFP–$Pin1^{S16E}$ did not bind phosphoproteins, as shown (Lu *et al.*, 1999b). Note that GFP fusion proteins were used because the WW domain Pin1 mutants were not stable in cells, but they were stable as GFP fusion proteins, although expressed at reduced levels (data not shown). Although the absolute maximal luciferase activity was not as high as in other experiments, which is likely to be due to lower levels of GFP fusion proteins being expressed, the overall trends were the same. (**C**) Inhibiting the ability of Pin1 to increase the c-Jun activity by DN-Ras. Cells were co-transfected with c-Jun or c-Jun + Pin1, and increasing amounts of DN-Ras, and then subjected to the luciferase assay. (**D** and **E**) Abolishing the cooperative effect between Pin1 and Ha-Ras or activated JNK by mutating c-Jun phosphorylation sites $Ser^{63/73}$. HeLa cells were co-transfected with various amounts of Pin1, c-Jun or $c\text{-}Jun^{S63/73A}$ construct, and Ha-Ras (D) or activated JNK (E) and then subjected to the luciferase assay.

the same target c-Jun. However, when the AP-1 site mutant cyclin D1 promoter was used in the same assay, only ≤10% of the transactivation measured for the wild-type promoter was observed (Figure 5E and F), indicating that transactivation of the cyclin D1 promoter by c-Jun, activated by Pin1, JNK or Ras, is dependent on the intact

AP-1-binding site. These results indicate that Pin1 cooperates either with activated JNK or oncogenic Ras to dramatically activate the cyclin D1 promoter. These cooperative effects are expected because Pin1 can regulate the transcriptional activity of c-Jun only after it has been phosphorylated by them.

To examine whether endogenous Pin1 is important for Ha-Ras to increase the transcriptional activity of c-Jun, we used $Pin1^{AS}$ to reduce cellular Pin1 levels (Figure 3B). When c-Jun and Ha-Ras were co-transfected with different concentrations of the $Pin1^{AS}$ construct, the transcriptional activity of c-Jun decreased significantly in a concentration-dependent manner (Figure 5A), indicating that inhibiting endogenous Pin1 decreases the ability of phosphorylated c-Jun to activate the cyclin D1 promoter. These results indicate that Pin1 cooperates with Ha-Ras or activated JNK to increase the activity of c-Jun toward the cyclin D1 promoter.

Pin1 contains a WW domain and a PPIase domain, which bind and isomerize specific pSer/Thr-Pro motifs, respectively, and both these activities are normally required for Pin1 to modulate the function of its phosphoprotein substrates, such as Cdc25C and tau (Ranganathan *et al.*, 1997; Yaffe *et al.*, 1997; Shen *et al.*, 1998; Lu *et al.*, 1999a,b). To examine whether only one, or both, of these activities is required for Pin1 to modulate the activity of c-Jun we used Pin1 mutants, $Pin1^{R68,69A}$, $Pin1^{W34A}$ and $Pin1^{S16E}$, which contain mutations at the key residues either in the PPIase domain (R68, R69) or the WW domain (W34 or S16), and fail to isomerize pSer/Thr-Pro bonds or to bind phosphoproteins (including c-Jun; data not shown), respectively (Shen *et al.*, 1998; Lu *et al.*, 1999b; Zhou *et al.*, 2000). In contrast to wild-type protein, these Pin1 mutants neither increased the transcriptional activity of c-Jun towards the cyclin D1 promoter nor cooperated with Ha-Ras to activate c-Jun (Figure 6A and B). Neither did the mutants affect the levels of c-Jun phosphorylation (data not shown). These results indicate that both phosphoprotein-binding and phosphorylation-specific isomerase activities of Pin1 are required for its ability to modulate the activity of c-Jun.

The above results suggest that Pin1 may increase the activity of c-Jun by binding and isomerizing its pSer/Thr-Pro motifs, as it does to Cdc25C and tau (Shen *et al.*, 1998; Lu *et al.*, 1999a; Zhou *et al.*, 2000). In this case, down-regulation of the Ras-dependent phosphorylation of c-Jun should reduce the effect of Pin1 on c-Jun, and mutations of the c-Jun phosphorylation sites that Pin1 binds to should abolish the Pin1 effect. To examine the first assumption, we co-transfected cells with Pin1, c-Jun and DN-Ras to examine the effect of DN-Ras on the ability of Pin1 to activate the cyclin D1 promoter. DN-Ras reduced both phosphorylation of c-Jun on $Ser^{63/73}$ and the ability of Pin1 to bind c-Jun (Figure 4A–D). Indeed, DN-Ras not only inhibited the ability of c-Jun to activate the cyclin D1 promoter, as shown previously (Albanese *et al.*, 1995), but also inhibited the ability of Pin1 to enhance the activity of c-Jun 5- to 7-fold (Figure 6C). These results suggest that the Ras-dependent phosphorylation of c-Jun is important for the Pin1 function on c-Jun. To examine the second assumption, we used the mutant $c\text{-}Jun^{S63/73A}$, which failed to bind Pin1 (Figure 4E and F). Pin1 almost completely failed to cooperate either with activated JNK or oncogenic Ha-Ras to increase the ability of $c\text{-}Jun^{S63/73A}$ to induce the cyclin D1 promoter (Figure 6D and E), indicating that phosphorylation of c-Jun on $Ser^{63/73}$ is essential for Pin1 to induce the cyclin D1 promoter. These results indicate that phosphorylation of c-Jun on $Ser^{63/73}$, induced by the Ras-dependent signaling pathway, is essential for Pin1 to increase transcription of the cyclin D1 promoter. Thus, Pin1 binds phosphorylated c-Jun and potentiates its transcriptional activity towards cyclin D1 in response to activation of Ras or JNK.

Discussion

Previous studies have demonstrated that depletion of Pin1 induces apoptosis and is also observed in neuronal cell death in Alzheimer's disease (Lu *et al.*, 1996, 1999a). We show here the striking overexpression of Pin1 in a large fraction of breast cancers. Furthermore, Pin1 levels correlate significantly with the grade of the breast tumors according, to Bloom and Richardson's classification system, although the relationship between Pin1 levels and the prognosis of cancer patients remains to be determined. Consistent with our findings is the observation that Pin1 is one of the genes that are most drastically suppressed by up-regulation of Brca1, as detected in cDNA array screening and northern analysis (MacLachlan *et al.*, 2000). In addition, the level of Pin1 in breast cancer cell lines is much higher than that in either normal or non-transformed mammary epithelial cells. Although further studies are needed to elucidate the mechanisms leading to overexpression of Pin1, these results demonstrate for the first time that Pin1 is up-regulated markedly in many human tumor samples.

The significance of Pin1 overexpression in cancer is further substantiated by our findings that Pin1 cooperates with activated JNK or Ha-Ras in increasing the transcriptional activity of phosphorylated c-Jun to activate the cyclin D1 promoter. Overexpression of cyclin D1 is found in 50% of patients with breast cancer (Bartkova *et al.*, 1994; Gillett *et al.*, 1994). Furthermore, overexpression of cyclin D1 contributes to cell transformation (Hinds *et al.*, 1994), whereas inhibition of cyclin D1 expression by antisense expression causes growth arrest of tumor cells (Schrump *et al.*, 1996; Arber *et al.*, 1997; Driscoll *et al.*, 1997; Kornmann *et al.*, 1998). Disruption of the cyclin D1 gene in mice blocks the proliferation of breast epithelial cells and reduces tumor development in response to Ha-Ras (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998). These results indicate that cyclin D1 plays an important role during oncogenesis, especially during Ras-mediated tumorigenesis (Rodriguez-Puebla *et al.*, 1999). Oncogenic Ras induces the cyclin D1 promoter via its AP-1 site (Albanese *et al.*, 1995). Although the AP-1 complex is composed of the c-Jun and c-Fos proteins, c-Jun is the most potent transactivator in the complex (Angel *et al.*, 1989; Chiu *et al.*, 1989; Abate *et al.*, 1991) and is elevated in Ha-Ras-transformed cells, in which c-Fos is down-regulated (Thomson *et al.*, 1990; Binetruy *et al.*, 1991). In addition to the regulation of protein levels, the activity of c-Jun is enhanced by phosphorylation induced by growth factors, oncogenic proteins, or stress conditions. Although different pathways may be involved, they eventually lead to activation of JNKs, which

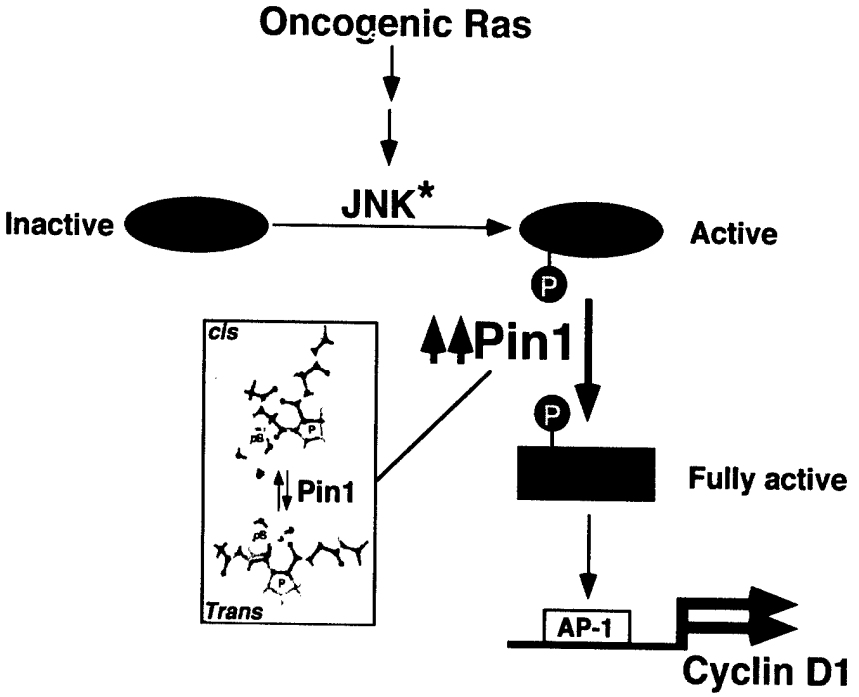


Fig. 7. Role of Pin1 in regulating the transcriptional activity of phosphorylated c-Jun towards the cyclin D1 promoter. Oncogenic Ha-Ras activates JNKs, which phosphorylate c-Jun on two critical amino terminal Ser-Pro motifs, enhancing its transcriptional activity. Pin1 is up-regulated in breast cancer and functions as a potent regulator of phosphorylated c-Jun to induce cyclin D1 expression, presumably by altering the conformation of the phosphorylated Ser-Pro motifs (insert). Double arrows, up-regulation; the asterisk indicated the activated form of proteins.

phosphorylate c-Jun on two critical N-terminal Ser-Pro motifs ($S^{63/73}$–P) and enhance its transcriptional activity (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Hunter and Karin, 1992; Derijard *et al.*, 1994; Hinds *et al.*, 1994; Albanese *et al.*, 1995, 1999; Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Whitmarsh and Davis, 1996; Karin *et al.*, 1997; Robles *et al.*, 1998; Bakiri *et al.*, 2000). Thus, phosphorylation of c-Jun on $Ser^{63/73}$-Pro is a key regulatory mechanism that converts inputs from various signaling pathways into changes in gene expression. However, it has not been described previously whether the activity of phosphorylated c-Jun is further regulated after phosphorylation.

We have found that Pin1 not only binds phosphorylated c-Jun, but also dramatically increases its ability to activate the cyclin D1 promoter in cooperation either with activated JNK or oncogenic Ha-Ras. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun, indicating that endogenous Pin1 is also required for the optimal activation of c-Jun. The significance of this Pin1-dependent regulation is further substantiated by our findings that up-regulation of Pin1 not only correlates with cyclin D1 overexpression in breast cancer tissues, but also induces cyclin D1 expression in breast cancer cell lines. Thus, Pin1 is a potent modulator of phosphorylated c-Jun in inducing cyclin D1 expression, presumably by regulating the conformation of the phosphorylated Ser-Pro motifs in c-Jun (Figure 7). The importance of Pin1 in the regulation of cyclin D1 expression has been further supported by our recent identification of cyclin D1 as one of the Pin1-induced genes in breast cancer cells in the differential display screen (Ryo *et al.*, 2001), and by our phenotypic analysis of Pin1-deficient mice (Y.-C.Liou, A.Ryo, H.K.Huang, P.J.Lu, F.Fujimori, T.Uchida, R.Bronson, T.Hunter and K.P.Lu, submitted). Although $Pin1^{-/-}$ mice have previously been shown to develop normally (Fujimori *et al.*, 1999), we have uncovered that they display a range of cell proliferative abnormalities, including decreased body size, retinal degeneration and neurological abnormalities. Moreover, in Pin1-deficient adult females, the breast epithelial compartment failed to undergo the massive proliferative changes caused by pregnancy (Y.-C.Liou, A.Ryo, H.K.Huang, P.J.Lu, F.Fujimori, T.Uchida, R.Bronson, T.Hunter and K.P.Lu, submitted). Significantly, many features of these Pin1-deficient mice, such as retinal degeneration and mammary gland impairment, are also characteristic of cyclin D1-deficient mice (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Moreover, cyclin D1 levels were significantly reduced in Pin1-deficient retina and breast epithelial cells from pregnant mice (Liou *et al.*, submitted). These results provide the genetic evidence for an essential role of Pin1 in maintaining cell proliferation and cyclin D1 expression, and further support a role of Pin1 in oncogenesis. Abnormal activation of the Ras-dependent signaling pathway and cyclin D1 overexpression are a common and critical mechanism during the development of many malignancies, such as breast, skin and colon cancer (Fantl *et al.*, 1995; Sicinski *et al.*, 1995; Robles *et al.*, 1998; Rodriguez-Puebla *et al.*, 1999). Indeed, Pin1 is significantly overexpressed in many of these human tumors (G.M.Wulf and K.P.Lu, unpublished data), suggesting that it plays a positive role for cell proliferation during oncogenesis (Figure 7).

In summary, our results show that Pin1 is strikingly overexpressed in human breast cancer tissues, and

cooperates with activated Ras signaling in increasing c-Jun transcriptional activity towards the cyclin D1 gene. Given the well established role of activated Ras signaling and cyclin D1 overexpression during oncogenesis, our study suggests that overexpression of Pin1 may promote tumor growth. In addition, since inhibition of the Pin1 enzymatic activity triggers tumor cells to enter apoptosis, overexpressed Pin1 may act as a novel anti-cancer target.

Materials and methods

Analysis of protein and mRNA levels in patient samples

Fifty-one cancerous and 10 normal breast tissue specimens were randomly selected. The malignancy of infiltrating carcinomas was scored according to Bloom and Richardson's classification system (Bloom and Richardson, 1957). Tissue from the core of the tumor was snap frozen in liquid nitrogen and pulverized using a Microdismembrator (Braun). About 10 μg of the pulverized tissues were resuspended in 100 μl of SDS sample buffer. Immunoblotting with anti-Pin1, anti-cyclin D1, anti-Her2/neu and anti-actin antibodies was performed as described (Shen *et al.*, 1998; Lu *et al.*, 1999a), as was immunohistochemistry using anti-Pin1 polyclonal or monoclonal antibodies (Lu *et al.*, 1999a). Levels of Pin1 and actin were semi-quantified using Imagequant, as described (Lu *et al.*, 1999a). mRNA was isolated using the Trizol reagent (Gibco) and cDNA was synthesized using Superscript (Gibco). Twenty-five nanograms of cDNA were used per real-time PCR run with primers specific for cyclin D1, and GAPDH as an internal control. All real-time PCR runs were performed in duplicate and analyzed according to the manufacturer's instructions (Applied Biosystems). The significance of the differences in Pin1 levels between clinical and pathological categories was analyzed using the Kruskal–Wallis test (Glantz, 1997). The Pearson correlation coefficients were obtained using the SAS software (Release 6.12; SAS Institute Inc., Cary, NC).

Determination of Pin1 levels and the effects of Pin1 on cyclin D1 expression in cell lines

The levels of Pin1 in normal (76N), spontaneously immortalized but not transformed (184B5 and MCF10), and transformed (MCF7, T47D, MDAMB435 and HCC1937) mammary epithelial cell lines were determined by subjecting total cellular proteins to immunoblotting analysis with anti-Pin1 polyclonal antibodies. To examine the nature of the double band, a tumor lysate was incubated at 30°C for 60 min in the presence of 100 nM okadaic acid (Sigma), PP1 and PP2A (Upstate Biotechnology) or CIP. To examine the effects of Pin1 on cyclin D1 expression, Pin1 cDNA was subcloned into pcDNA3 vector (Invitrogen) and transfected into MCF7, T47D or HeLa cells for 36 h, followed by determining the level of Pin1 and cyclin D1 by immunoblotting analysis with anti-Pin1 and anti-cyclin D1 antibodies, respectively, as described (Lu *et al.*, 1996; Shen *et al.*, 1998), and cyclin D1 mRNA by real-time PCR, as described above.

Determination of the Pin1-c–Jun interaction

To examine the interaction between Pin1 and phosphorylated c-Jun, HeLa cells were co-transfected with c-Jun or c-Jun$^{S63/73A}$ and the oncogenic Ha-Ras, consititutively active JNK, DN-Ras or the control vector for 24 h. The cells were lysed in a lysis buffer containing 1% Triton X-100, and the supernatants incubated with 10 μl of agarose beads containing various GST–Pin1 proteins or control GST for 2 h at 4°C. The precipitated proteins were washed five times in the buffer containing 1% Triton X-100 before being subjected to immunoblotting analysis using antibodies against c-Jun or c-Jun phosphorylated on Ser$^{63/73}$ (New England Biolabs), as described (Yaffe *et al.*, 1997; Shen *et al.*, 1998; Lu *et al.*, 1999a,b). For co-immunoprecipitation, we used anti-c-Jun polyclonal antibodies (Santa Cruz) and unrelated polyclonal antibodies (Pericentrin antibodies) as a control. The pre-cleared lysates were incubated for 2 h with the respective antibodies, and the immune complexes were collected with protein A beads (Sigma) and subjected to immunoblotting with anti-Pin1 or anti-c-Jun antibodies. The ability of the Pin1 WW domain and PPIase domain mutants to bind phosphoproteins (MPM-2 or c-Jun) and to isomerize pSer/Thr-Pro bonds were determined, as described (Yaffe *et al.*, 1997; Lu *et al.*, 1999a,b).

Promoter reporter assays

Various cyclin D1–luciferase reporter constructs, c-Jun and Ras constructs were gifts from R.Pestell (Albert Einstein College of Medicine), M.Karin (University of California at San Diego) and L.Feig (Tufts University), respectively, and have been confirmed by DNA sequencing. Luciferase reporter constructs for TK, c-fms and MMTV were purchased. Superfect (Qiagen) was used for transfections. Reporter gene assays were performed with the Dual-luciferase reporter assay system (Promega) at 24–36 h after transfection. One nanogram of pRL-TK (Promega) *Renilla* luciferase was co-transfected in each sample as an internal control for transfection efficiency. Expression of all transfected genes was confirmed by immunoblotting analysis with the respective antibodies. The amounts of DNA used in transfection were carefully titrated for each construct; typically, only ~50 ng of each DNA were used, with exceptions indicated in the text. The activity of the reporter luciferase was expressed relative to the activity in control vector-transfected cells, which was defined as 1. Similar results were obtained in at least three different experiments. All results are expressed as $\bar{x} \pm$ SD of independent duplicate cultures. Since Pin1^{AS} induces mitotic arrest and apoptosis at 48–72 h after transfection (Lu *et al.*, 1996), all experiments with Pin1^{AS} were performed before 36 h, when no significant apoptotic cells were observed, as described previously (Lu *et al.*, 1996).

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Telomeric protein Pin2/TRF1 induces mitotic entry and apoptosis in cells with short telomeres and is down-regulated in human breast tumors

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Telomeres are essential for cell survival and have been implicated in the mitotic control. The telomeric protein Pin2/TRF1 controls telomere elongation and its expression is tightly regulated during cell cycle. We previously reported that overexpression of Pin2/TRF1 affects mitotic progression. However, the role of Pin2/TRF1 at the interface between cell division and cell survival remains to be determined. Here we show that overexpression of Pin2 induced apoptosis in cells containing short telomeres, but not in cells with long telomeres. Furthermore, before entering apoptosis, Pin2-expressing cells first accumulated in mitosis and strongly stained with the mitosis-specific MPM2 antibody. Moreover, Pin2-induced apoptosis is potentiated by arresting cells in mitosis, but suppressed by accumulating cells in G1. In addition, overexpression of Pin2 also resulted in activation of caspase-3, and its proapoptotic activity was significantly reduced by inhibition of caspase-3. These results indicate that up-regulation of Pin2/TRF1 can specifically induce entry into mitosis and apoptosis, likely via a mechanism related to activation of caspase-3. Significantly, we also found that, out of 51 human breast cancer tissues and 10 normal controls examined, protein levels of Pin2/TRF1 in tumors were significantly lower than in normal tissues, as detected by immunoblotting analysis and immunocytochemistry. Since down-regulation of Pin2/TRF1 allows cells to maintain long telomeres, these results suggest that down-regulation of Pin2/TRF1 may be important for cancer cells to extend their proliferative potential. *Oncogene* (2001) **20,** 1497–1508.

Keywords: apoptosis; cancer; cell cycle; pin2/TRF1; telomeres; telomeric protein

Introduction

Telomeres are essential for preserving chromosome integrity during cell division. Telomeres are composed of repetitive DNA sequences of TTAGGG arrays concealed by a complex of telomeric proteins that protects the ends from exonucleolytic attack, fusion and incomplete replication (Greider and Blackburn, 1996; Lundblad, 2000; Zakian, 1995). There is growing evidence suggesting that both the shielding of telomeric ends and their elongation are dependent on telomere-binding proteins. For example, homeostasis of telomere length in budding and fission yeast cells requires the telomeric proteins Rap1p and Taz1, respectively (Cooper *et al.*, 1997; Krauskopf and Blackburn, 1996; Marcand *et al.*, 1997). Mutagenesis analyses of telomeric sequences of *Kluyveromyces lactis* also suggest that telomere length is modulated by proteins that bind to double strand telomeric DNA (McEachern and Blackburn, 1995). Telomere maintenance in mammalian cells is also regulated by telomere binding proteins, including TRF1 and TRF2 (Chong *et al.*, 1995; van Steensel and de Lange, 1997). TRF1 has been shown to negatively regulate telomere maintenance; overexpression of TRF1 accelerates telomere shortening, whereas dominant-negative TRF1 increases telomere elongation (van Steensel and de Lange, 1997). These results indicate that telomere-binding proteins play a pivotal role in telomere metabolism.

Several observations link telomeres to mitotic progression. In *Drosophila*, deletion of telomeres triggers mitotic arrest and apoptosis (Ahmad and Golic, 1999). In fission yeast, telomeres are clustered at the nuclear periphery in G2, but this association is disrupted in mitosis (Funabiki *et al.*, 1993), and telomeres have been shown to mediate the attachment of chromosomes to spindle bodies and lead chromosome movement in meiotic prophase (Chikashige *et al.*, 1994). In budding yeast, elimination of telomeres causes a Rad9p-mediated cell cycle arrest in G2 in budding yeast (Sandell and Zakian, 1993) and mutations in the related *TEL1* and MEC1 genes result in shortened telomeres, G2/M checkpoint defect and genomic instability (Greenwell *et al.*, 1995; Sanchez *et al.*, 1996). Similarly, mutations in its human counterpart, the *ATM* gene, causes ataxia-telangiectasia (AT) both in humans and mice, displaying a wide range of abnormalities, including those related to telomere dysfunction (Barlow *et al.*, 1996; Elson *et al.*, 1996; Savitsky *et al.*, 1995; Xu *et al.*, 1996; Xu and Baltimore, 1996). More interestingly, cell lines derived from AT patients have

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shortened telomere lengths (Metcalfe *et al.*, 1996; Pandita *et al.*, 1995; Xia *et al.*, 1996) and defected G2/M checkpoint (Beamish *et al.*, 1994; Rudolph and Latt, 1989). Finally, mutations in the *Tetrahymena* telomeric DNA sequence has been shown to cause a block in anaphase chromosome separation (Kirk *et al.*, 1997). Collectively, these results suggest that telomeres may be important for regulation of mitosis. However, little is known about the identity and function of the signaling molecule(s) involved in this process.

We independently isolated the telomere-binding protein Pin2 as a one of three proteins, Pin1–3, which interact with NIMA kinase that is an essential mitotic kinase in Aspergillus nidulans (Lu *et al.*, 1996). Characterization of these Pin proteins shows that they are all involved in mitotic regulation (Lu, 2000). Pin1 binds and regulates the function of a subset of phosphoproteins by controlling the conformation of specific phosphorylated Ser/Thr-Pro motifs (Lu *et al.*, 1999a,b; Shen *et al.*, 1998; Yaffe *et al.*, 1997; Zhou *et al.*, 2000). Pin2 is identical in the sequence to TRF1 apart from an internal deletion of 20 amino acids (Shen *et al.*, 1997). TRF1 and Pin2 are likely two alternatively spliced isoforms of the same gene *PIN2/TRF1*, as suggested by Young *et al.* (1997). For clarity, we will here use Pin2 for the 20 amino acid deletion isoform and TRF1 for the 20 amino acid containing isoform, as they were originally identified (Chong *et al.*, 1995; Shen *et al.* 1997), and will refer to the endogenous Pin2 and TRF1 proteins as Pin2/TRF1 since it is difficult to physically or functionally separate these isoforms at the present time. However, we have shown that Pin2 is 5–10-fold more abundant than TRF1 in the cells and the expression level of Pin2/TRF1 is tightly regulated during the cell cycle (Shen *et al.*, 1997). Both Pin2 and TRF1 contain a D-like motif similar to the destruction box present in many mitotic proteins, and their protein levels are significantly increased in late G2 and mitosis and then degraded as cells exit from mitosis. Furthermore, overexpression of Pin2 or TRF1 resulted in accumulation of the cells in G2 or M phase of the cell cycle (Shen *et al.*, 1997). Although these results together suggest that Pin2/TRF1 may affect mitotic progression, it is not clear whether Pin2/TRF1 has any specific effect on mitosis. Furthermore, it is unknown why overexpression of Pin2/TRF1 has no effect on the cell cycle in some cells (van Steensel and de Lange, 1997).

Here, we describe that overexpression of Pin2 induced entry into mitosis and apoptosis only in cells with short telomeres, but not in cells with long telomeres. Moreover, Pin2-induced apoptosis is potentiated by mitotic arrest, but not suppressed by G1 arrest. Importantly, Pin2 overexpression induced activation of caspase-3, a key executioner of apoptosis, and its ability to induce apoptosis was significantly suppressed by inhibition of caspase-3. These results have demonstrated that Pin2/TRF1 can specifically affect the cell cycle, triggering mitotic entry and apoptosis in the cells containing short telomeres. Consistent with a potential role of Pin2/TRF1 in regulation of cell survival, we found that protein levels of Pin2/TRF1 were significantly reduced in human breast cancer tissues, as compared with those in normal controls. Since down-regulation of Pin2/TRF1 allows cells to maintain long telomeres (van Steensel and de Lange, 1997), these results suggest that down-regulation of Pin2/TRF1 may be important for cancer cells to divide continuously.

Results

Overexpression of Pin2 induces apoptosis in HeLa cells

We previously showed that ectopic expression of Pin2 or TRF1 in HeLa cells caused accumulation of cells with 4N DNA content (Shen *et al.*, 1997), which is normally observed in G2 and/or M phase of the cell cycle. To further characterize this Pin2/TRF1-induced phenotype, we transfected cDNA constructs for Pin2 and TRF1 into HeLa cells and A-T22IJE-T. A-T22IJE-T cells were originally derived from primary A-T fibroblasts and contain no ATM protein (Ziv *et al.*, 1989). After transient expression, two proteins were detected in the nucleus of cells 8 h after transfection by indirect immunofluorescence. However, cells expressing Pin2 or TRF1 rounded up and died after 28–32 h, and a few cells were detectable at 72 h, suggesting that Pin2 and TRF1 were toxic to the cells. To visualize surviving Pin2/TRF1-expressing cells, we co-transfected cells with a β-gal expression construct. After co-transfection of HeLa or A-T22IJE-T cells with Pin2 or TRF1 and β-gal constructs, the number of X-gal-stained surviving blue cells was significantly reduced, as compared with that of empty vector- or antisense Pin2-transfected cells (Figure 1, data not shown). Since there was no detectable difference in phenotypic changes induced by Pin2 or TRF1 (Table 1), we focused on Pin2 for subsequent experiments.

The morphological changes of Pin2-expressing cells were suggestive of apoptosis. To confirm that overexpression of Pin2 indeed induces apoptosis, we first used the TUNEL assay, which detects apoptosis-specific DNA breaks (Douglas *et al.*, 1998; Gavrieli *et al.*, 1992). To detect transfected cells by flow cytometry, HeLa cells were co-transfected with CD20, as described (Zhu *et al.*, 1993). Whereas there were less than 0.4–1.0% TUNEL-positive cells in vector-transfected cells, about 15–20% of TUNEL-positive cells were detected in a Pin2-transfected cell population (data not shown). To directly observe morphological changes of Pin2-expressing cells, we inserted green fluorescence protein (GFP) at the NH_2-terminal end of the Pin2 cDNA. After 28 h transfection, 38% of GFP-Pin2-expressing HeLa cells and 56% of GFP-Pin2-expressing A-T22IJE-T rounded up and the condensed chromatin became fragmented, with the formation of micronuclei (Figure 2a, also see 4a), which are characteristic of apoptotic cells. When Pin2-transfected cells were subjected to flow cytometry after staining with propidium iodide, as shown later in Figure 5b

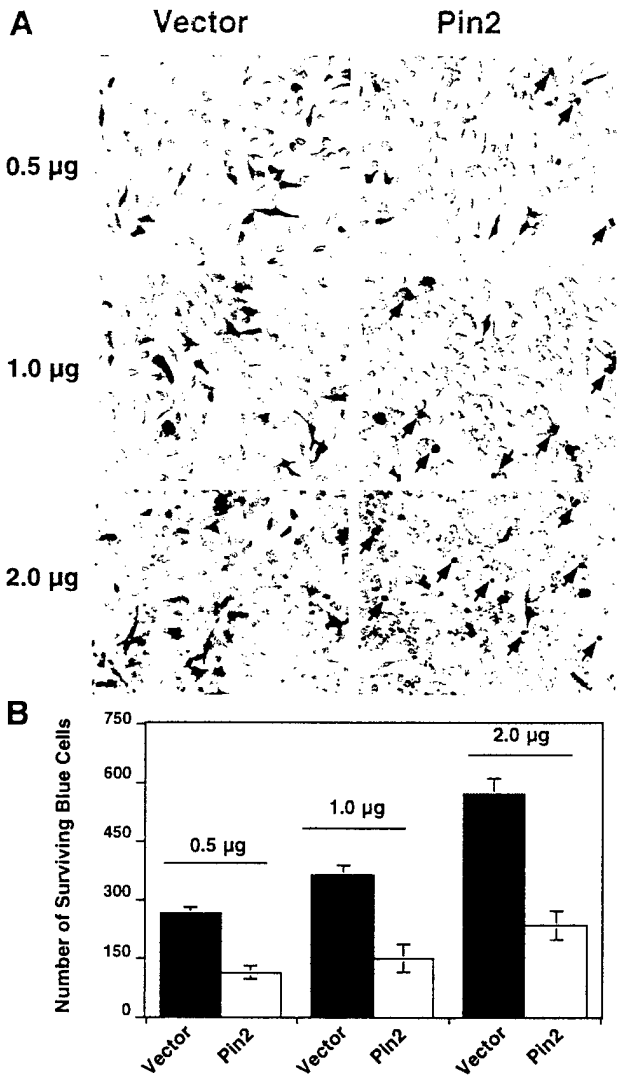

Figure 1 Expression of Pin2/TRF1 affects cell viability in a concentration-dependent manner. HeLa cells were cotransfected with different amounts of the Pin2/TRF1 expression construct or control vector, together a pSV2-lacZ reporter construct. Cells were fixed at 60 h after transfection, stained with X-gal and examined microscopically (**a**). Arrows point to dead cells. The number of surviving blue cells were counted and presented in (**b**)

about 25% of GFP-Pin2-expressing HeLa cells contained a sub-G1 DNA content, which is another feature of apoptosis. These multiple assays confirmed that ectopic expression of Pin2 can induce apoptosis in HeLa and A-T22IJE-T cells.

The ability of Pin2 to induce apoptosis depends on the concentration of unbound Pin2 and telomere length in the cell

After establishing that Pin2/TRF1 induces apoptosis, we examined its relationship to telomeres. Since the C-terminal Myb-type DNA-binding domain of Pin2/TRF1 binds telomeric DNA repeats and is also required for Pin2/TRF1 to inhibit telomere elongation and to affect mitotic progression (Shen *et al*., 1997; van Steensel and de Lange, 1997), we examined whether this domain is required for Pin2 to induce apoptosis. Although two C-terminal truncation mutants, $Pin2^{1-372}$ and $Pin2^{1-316}$ were expressed in cells at levels that were similar to that of wild-type protein, they were not localized at telomeres (Figure 2), as shown previously (Shen *et al*., 1997; van Steensel and de Lange, 1997). In contrast to wild-type Pin2, neither of the truncation mutants induced apoptosis in HeLa (data not shown) or A-T22IJE-T cells and less than 10% of transfected cells were apoptotic (Figure 2a–c). These results indicate that the full-length Pin2, including telomeric DNA-binding domain is required for apoptosis induction. To further examine whether the ability of Pin2 to induce apoptosis depends on the actual binding of the full-length Pin2 to the telomeric DNA, we introduced triple Ala substitutions into three residues in Pin2, Lys401, Asp402 and Arg403. These three residues are highly conserved in Myb-type DNA-binding domains of telomeric proteins and are also involved in binding telomeric DNA, as revealed by determining the crystal structure of the DNA-binding domain of yeast RAP1 in complex with telomeric DNA (Konig *et al*., 1996). When the triple Pin2 mutant ($Pin2^{3A}$) expression construct was transfected into HeLa cells, the mutant protein was detected in the nucleus, but not at telomeres (Table 1), as expected. However, $Pin2^{3A}$ still induced apoptosis (Table 1). These results indicate that apoptosis is induced by the full-length Pin2 protein, but its telomeric binding is not required, suggesting that apoptosis is likely due to a high concentration of unbound Pin2.

Since apoptosis induced by inhibition of telomerase depends on telomere length (Hahn *et al*., 1999; Herbert *et al*., 1999; Zhang *et al*., 1999), we asked whether telomere length affected the ability of Pin2 to induce apoptosis. To address this question, we compared the ability of Pin2 to induce apoptosis in six cell lines with different telomere lengths, including HeLa1.2.11, which is derived from HeLa cells. In contrast to most HeLa cells that contain short telomeres (1–3 kb), HeLa1.2.11 cells have rather long telomeres (15–30 kb) (Table 1) (Ishibashi and Lippard, 1998). Interestingly, Pin2 potently induced the apoptotic phenotype in three different cell lines, HeLa, A-T22IJE-T and A431, all of which contain short telomeres (Figure 3a, Table 2) (Metcalfe *et al*., 1996; Pandita *et al*., 1995; Xia *et al*., 1996; Zhang *et al*., 1999). In contrast, Pin2 almost completely failed to induce significant apoptosis in three other cell lines, HeLa1.2.11 and 293 and HT1080, which contain long telomeres (Table 2) (Ishibashi and Lippard, 1998; van Steensel and de Lange, 1997; Zhang *et al*., 1999). For example, in HeLa1.2.11 cells, expressed GFP-Pin2 was highly concentrated at telomeres, displaying a very prominent speckled pattern without affecting cell viability (Figure 3b), which is consistent with the fact that these cells have long telomeres (Ishibashi and Lippard, 1998). These results show that the ability of Pin2 to induce apoptosis at least in part depends on

Table 1 Induction of apoptosis by Pin2 and its mutants

Pin2 construct		Telomeric binding	Apoptosis (%)
Vector	GFP	-	5.4±2.2
Pin2	GFP, NLS DB, 1–419	+	37.6±8.9
$Pin2^{1-372}$	GFP, 372	-	6.8±2.4
$Pin2^{1-316}$	GFP, 316	-	6.2±1.9
$Pin2^{3A}$	GFP, AAA	-	34.3±7.3
TRF1	GFP, I, 439	+	32.3±5.6

Expression constructs expressing GFP-Pin2 or TRF1 or its various mutants were transfected into HeLa cells. Cells were fixed at 28 h after transfection, and stained with DAPI, followed by determining percentage of cells displaying apoptotic phenotype in at least 300 GFP-positive cells. The results represent the mean ± s.d. of at least three experiments. The telomeric binding of TRF1, Pin2 and its truncation mutants was reported previously (Shen *et al.*, 1997) and the same method was used to determine the telomeric binding of $Pin2^{3A}$. NH_2-terminal GFP box, GFP epitope tag inserted at the NH2-terminus; DB, the Myb-type DNA-binding domain that binds the telomeric DNA repeats; NLS, nuclear localization signal; I, 20 amino acid insert unique to TRF1

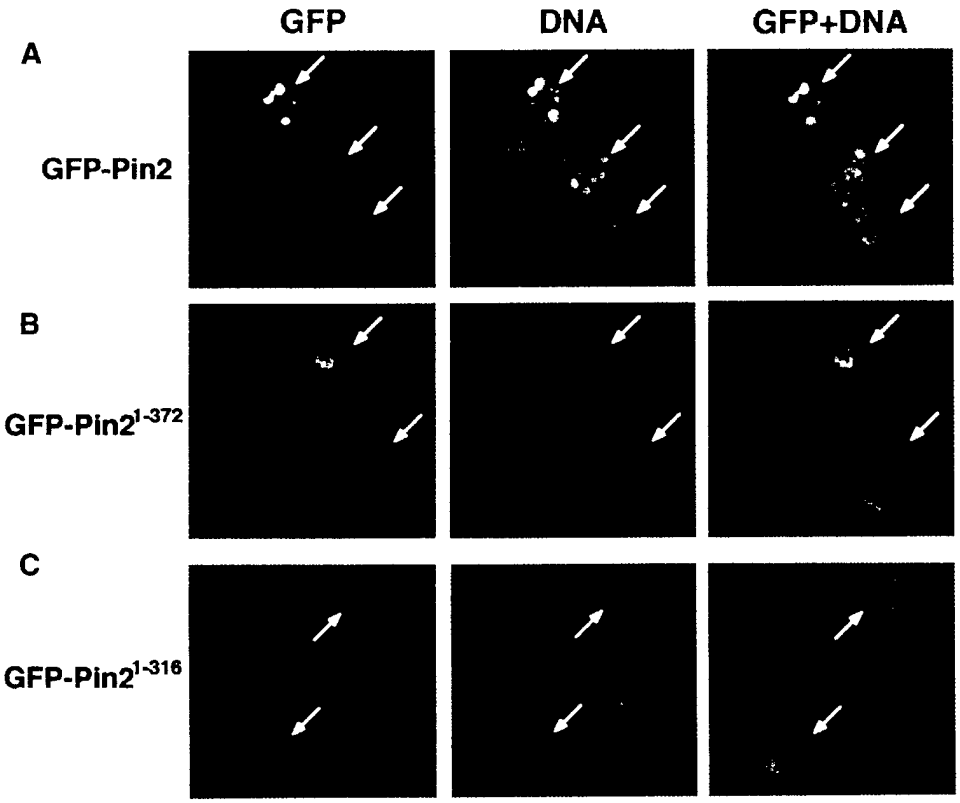

Figure 2 Expression of Pin2, but its telomere-binding mutants, induces apoptosis. A-T22IJE-T and HeLa cells were transfected with expression constructs encoding GFP-Pin2 (**a**) or its COOH-terminal truncation mutants, GFP- $Pin2^{1-372}$ (**b**) or - $Pin2^{1-316}$ (**c**). Cells were fixed at 30 h after transfection and stained with DAPI. Similar results were also obtained in HeLa cells (not shown). Arrows point to transfected cells. GFP alone did not induce apoptosis (not shown)

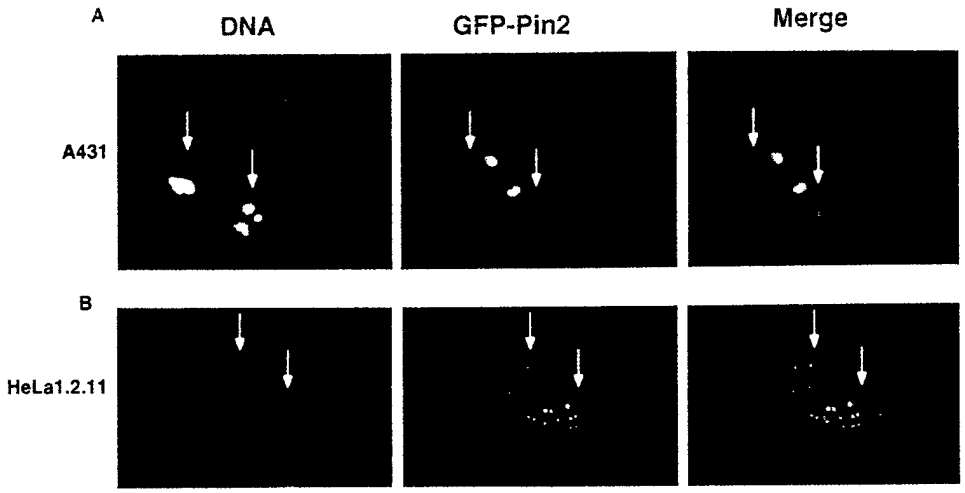

Figure 3 Expression of Pin2 induces apoptosis in A431 cells, but not in HeLa1.2.11 cells. A431 (**a**) and HeLa1.2.11 (**b**) cells were transfected with a GFP-Pin2 construct or the control GFP vector for 28 h, then fixed and stained with DAPI, followed by microscopy. Arrows point to Pin2-expressing cells. GFP alone did not induce apoptosis (data not shown)

Table 2 Pin2 induces apoptosis in cells with short telomeres, but not in cells with long telomeres

Cell line	*Telomere length*	*Pin2-induced apoptosis*
HeLa	Short (1–3 kb)[a]	+
A-T22IJE-T	Short (1–4 kb)[b]	+
A431	Short (1–3 kb)[c]	+
HeLa1.2.11	Long (15–30 kb)[d]	–
293	Long (6–12 kb)[c]	–
HT1080	Long (4–9 kb)[e]	–

Various cell lines were transfected with GFP-Pin2 expression construct or control GFP vector for 30 h and then fixed and stained with DAPI, followed by determining percentage of cells displaying apoptotic phenotype in total GFP-positive cells. [a]Telomere length in HeLa cells used was about 1–3 kb, determined as described in Materials and methods (data not shown); [b]Telomere length in all A–T cell lines examined is reported to be 1–4 kb (Pandita *et al.*, 1995; Xia *et al.*, 1996; Metcalfe *et al.*, 1996), although telomere length in A-T22IJE-T cell line has not been specifically determined; [c]Telomere length in A431 and 293 is reported to be 1–3 and 10–12 kb respectively (Zhang *et al.*, 1999); [d]Telomere length in HeLa1.2.11 is reported to be 15–30 kb (Ishibashi and Lippard, 1998); [e]Telomere length in HT1080 is reported to be 4–9 kb (van Steensel and de Lange, 1997); +, Apoptotic phenotype is easily detected in 30–55% of GFP-Pin2-expressing cells; –, Apoptotic phenotype is detected below 5–12% of GFP-Pin2-expressing cells, which were similar to control GFP-expressing cells

telomere length. These results are also consistent with our findings that the ability of Pin2 to induce apoptosis depends on the concentration of unbound Pin2.

Overexpression of Pin2 induces mitotic entry and apoptosis

To examine whether Pin2 specifically affects cell cycle progression before inducing apoptosis, we performed a detailed time course analysis of morphological changes of Pin2-transfected cells. At 12–16 h after transfection, GFP-Pin2 was primarily localized in the nucleus with punctate speckles during interphase, and was concentrated at telomeres with some diffuse staining all over the cells in the mitotic stage (Figure 4a). These patterns of the localization were similar to those of endogenous Pin2/TRF1 (Shen *et al.*, 1997; van Steensel and de Lange, 1997), indicating that GFP does not affect the localization of the Pin2 proteins. Importantly, the number of mitotic cells was significantly increased in the population of GFP-Pin2-expressing cells during the period of 20–24 h after transfection (Figure 4a,b). However, these Pin2-transfected cells apparently did not progress through normal mitosis. Instead, the condensed chromatin eventually seemed to become fragmented and micronuclei were formed (Figure 4a,b), a phenotype characteristic of apoptotic cells. These results suggest that ectopic expression of Pin2 leads to entry into mitosis, which is followed by apoptosis.

If Pin2 first induces mitotic entry and then apoptosis, there are at least two predictions. First, Pin2-induced apoptotic cells would have some mitosis-specific markers and, second, Pin2-induced apoptosis would be increased if cells are arrested at mitosis by other approaches, but decreased if cells are not allowed to enter mitosis. To examine the first prediction, we stained Pin2-transfected cells with the phospho-specific MPM-2 monoclonal antibody because MPM-2 specifically recognizes a subset of mitosis-specific phosphoproteins and has been widely used as a maker for mitotic cells (Davis *et al.*, 1983; Matsumoto-Taniura *et al.*, 1996; Vandre *et al.*, 1986; Westendorf *et al.*, 1994; Yaffe *et al.*, 1997). As shown in Figure 4c, most Pin2-expressing cells were strongly stained with MPM-2 20–24 h after transfection. This staining was also observed in Pin2-expressing apoptotic cells even 28–32 h after transfection, although at a weaker intensity (Figure 4c). This, however, is expected because it is impossible to maintain the high level of mitotic phosphorylation at such a stage of apoptosis. These results confirm that

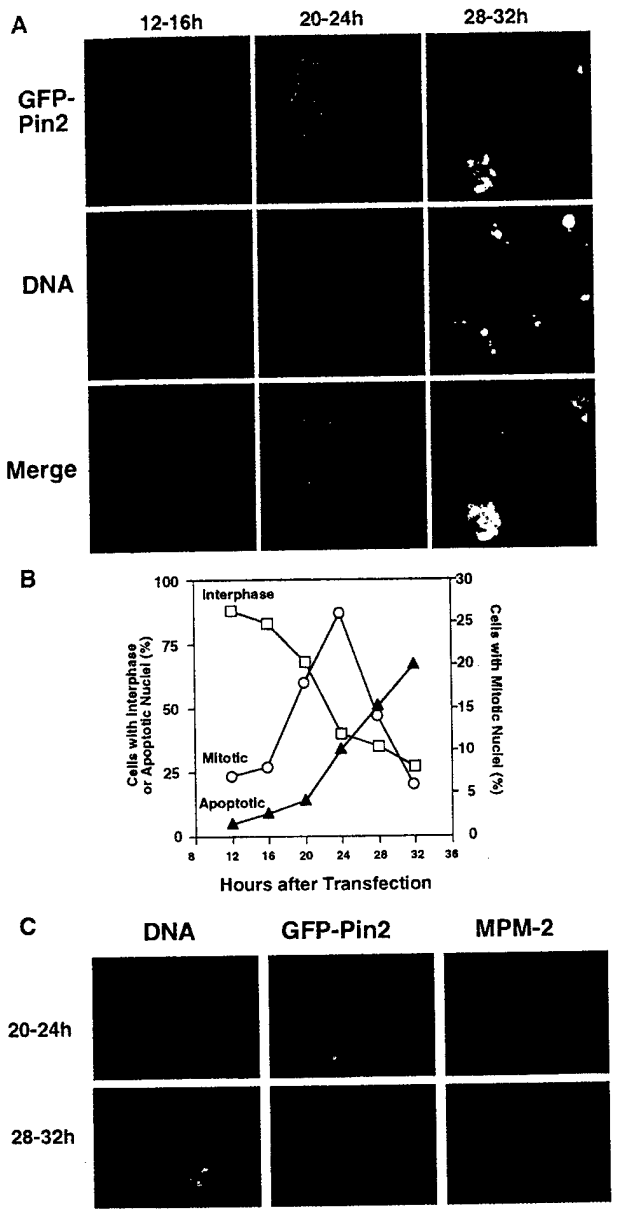

Figure 4 Expression of Pin2 induces mitotic entry and apoptosis in HeLa cells. After transfection with GFP-Pin2 construct, HeLa cells were monitored over time and fixed at times indicated, stained with DAPI and microscopically examined (**a**). The cells with interphase, mitotic or apoptotic nuclear morphology in all GFP-Pin2-expressing cells were counted (**b**). Cells were stained with MPM-2 antibody, followed by fluorescence microscopy (**c**)

Pin2-expressing cells are accumulated at mitosis before entering apoptosis.

To examine the second prediction, we transfected HeLa cells with GFP-Pin2 or membrane-localized control GFP (TM-GFP) for 12 h and then treated the cells with nocodazole or lovastatin, followed by analysing the cell cycle profiles of transfected and non-transfected cells using flow cytometry analysis. The membrane-localized TM-GFP was used instead of GFP because regular GFP leaked out of cells after fixing with ethanol for flow cytometric assay. When cells were transfected with TM-GFP, both GFP-positive and -negative cells were almost completely arrested at mitosis after nocodazole treatment. Lovastatin treatment significantly increased G1 cells and reduced S phase cells, although the arrest was not complete (Figure 5a), as shown previously (Jakobisiak *et al*., 1991; Keyomarsi *et al*., 1991). This is likely due to the difficulty in completely synchronizing HeLa cells

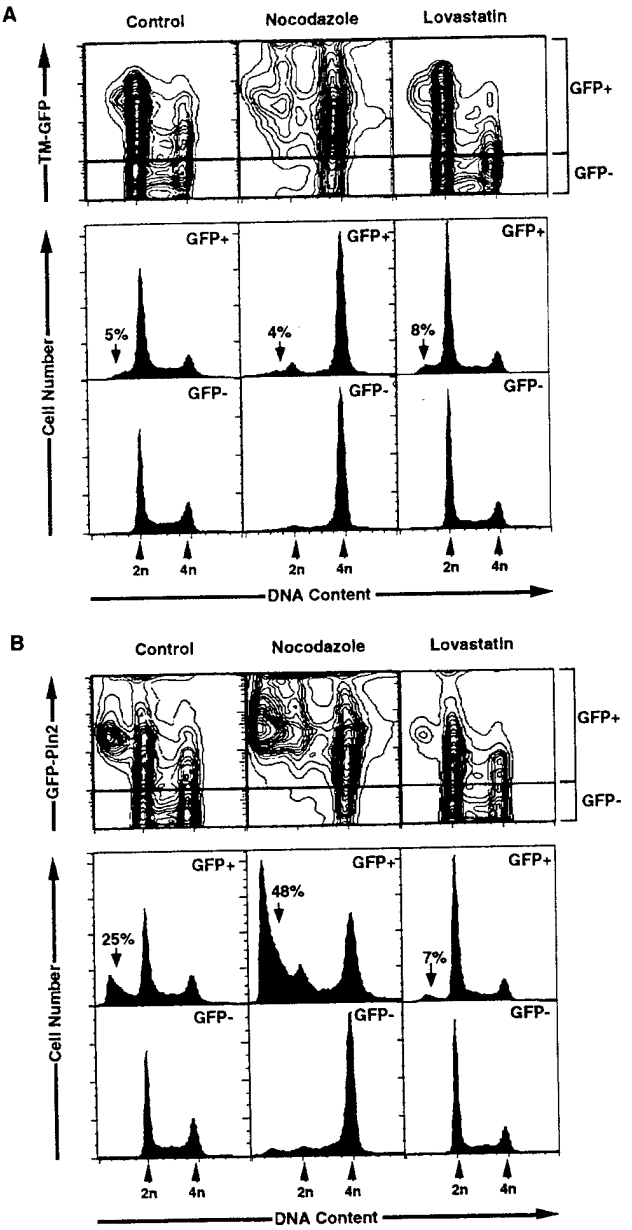

Figure 5 Pin2-induced apoptosis is potentiated by arresting cells at mitosis, but suppressed by arresting cells in G1. HeLa cells were transfected with membrane-localized TM-GFP vector (**a**) GFP-Pin2 (**b**) for 12 h and treated with 100 ng/ml nocodazole for 16 h or 20 μM lovastatin for 20 h to accumulate cells at mitosis or G1, respectively. Cells were stained with propidium iodide and GFP-positive and -negative cells, separated and their cell cycle profiles analysed using flow cytometry. Percentages and arrows indicate apoptotic cells with the sub-G1 DNA content in total cells examined

(Keyomarsi *et al.*, 1991). Furthermore, sub-G1 apoptotic cells were rather minimal (Figure 5a). These results indicate that nocodazole and lovastatin do produce the expected cell cycle arrests, which are not significantly affected by GFP. When the cells were transfected with GFP-Pin2, the cell cycle profiles of the non-transfected cells were similar to those of the control cells (Figure 5b, GFP−). However, in GFP-Pin2-positive cells, about 25% of cells were apoptotic, as indicated by the sub-G1 DNA content (Figure 5b, GFP+). This Pin2-induced apoptosis was reduced to 7% after lovastatin treatment, but dramatically increased to 48% after nocodazole treatment (Figure 5b). These results indicate that Pin2-induced apoptosis is dramatically increased if cells are arrested at mitosis, but decreased if cells are arrested in G1. Taken together, the above results indicate that overexpression of Pin2 induces entry into mitosis and apoptosis.

Overexpression of Pin2 results in activation of capase-3

After establishing that overexpression of Pin2 induces mitotic entry and apoptosis, we asked whether Pin2 activates caspase-3, because during apoptosis caspase-3 is a key executioner that is responsible either partially or totally for the proteolytic cleavage of many essential proteins (Cryns and Yuan, 1998). Activation of caspase-3 requires proteolytic processing of its inactive form into activated two subunits, p17 and p12 (Nicholson *et al.*, 1995). Since cleaved caspase-3 antibodies can detect the fragments of activated caspase-3, it is possible to examine the activity of caspase-3 in cells using flow cytometry, as shown previously (Belloc *et al.*, 2000). To examine the effect of overexpression of Pin2 on activation of caspase-3, we transfected cells either with GFP-Pin2 or membrane targeted TM-GFP for 30 h. Cells were stained with the cleaved caspase-3 antibodies and immunoreactivity of GFP-positive and -negative cells was determined by flow cytometry. As shown in Figure 6a, the fluorescence intensity of cleaved caspase-3 was significantly increased in cells expressing GFP-Pin2, as compared with that of GFP-negative cells in the same transfection population. In contrast, the fluorescence intensity of cleaved caspase-3 showed little difference between TM-GFP-positive and -negative cells. These results indicate that caspase-3 is specifically activated in GFP-Pin2-transfected cells. To examine the significance of the capase-3 activation, we used the caspase-3 inhibitor DEVD-CHO. As shown in Figure 6b, the caspase-3 inhibitor significantly suppressed the ability of Pin2 to induce apoptosis. These results indicate that Pin2 is able to activate caspase-3 and that caspase-3 is a downstream mediator of Pin2-induced apoptosis.

Expression of Pin2/TRF1 is down-regulated in human breast cancer samples

It has been previously shown that overexpression of TRF1 induces telomere shortening (van Steensel and de Lange, 1997). We show that overexpression of Pin2 or TRF1 induces apoptosis. These results led us to suspect that expression of Pin2/TRF1 may be reduced in cancer, where telomeres have to be maintained and apoptosis is often inhibited. To examine this possi-

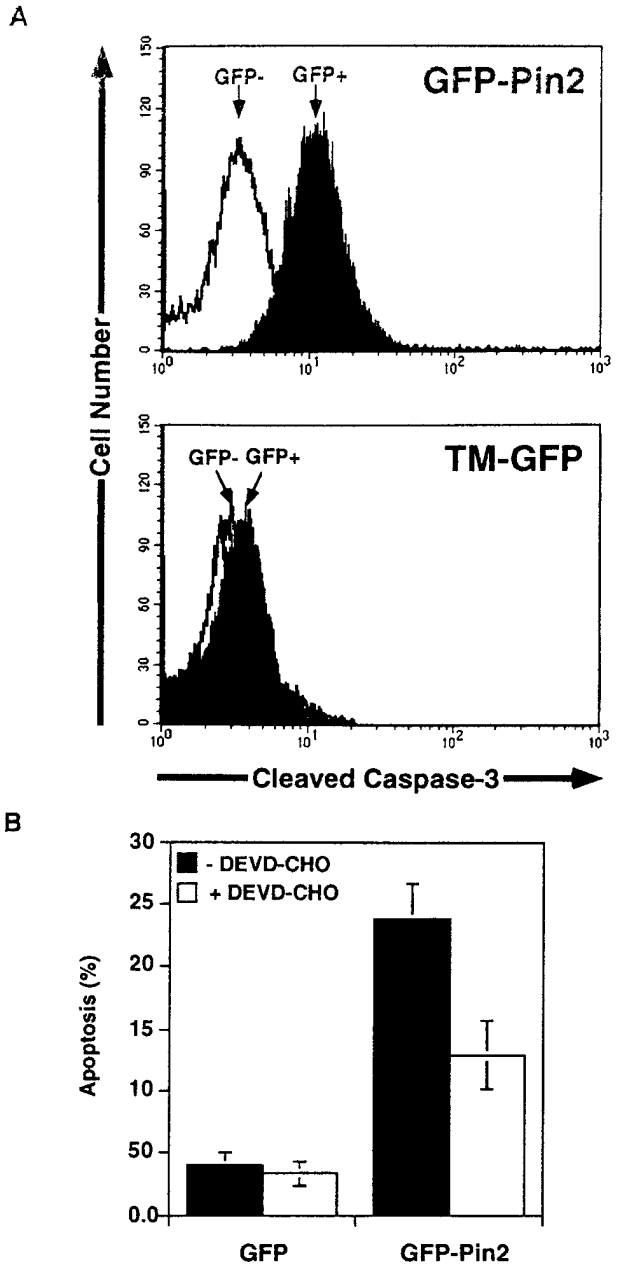

Figure 6 Overexpression of Pin2 leads activation of capase-3 and inhibition of capase-3 suppresses Pin2-induced apoptosis. (**a**) After 28 h of transfection with expression construct encoding GFP-Pin2 or the membrane targeted GFP (TM-GFP), HeLa cells were fixed and immunostained with cleaved caspase-3 antibodies and GFP-positive and -negative cells were sorted out and their immunoreactivity was determined by flow cytometry. Note, since regular GFP is leaked from cells after ethanol fixation, TM-GFP was instead used as a control. (**b**) Cells were pre-incubated with 10 μM of Ac-DEVD-CHO and then transfected with GFP-Pin2 or control GFP. Thirty hours later, cells were fixed and stained with DAPI, followed by determining percentage of apoptotic cells in total GFP-positive cells

bility, we compared protein levels of Pin2/TRF1 in normal human breast and breast cancer tissues using immunohistochemistry and immunoblotting with affinity-purified anti-Pin2/TRF1 antibodies. Pin2/TRF1 was readily detected in ductal epithelial cells, connective tissue and blood vessels in normal breast tissues (Figure 7a). Furthermore, Pin2/TRF1 staining was primarily in the nucleus (Figure 7a), as shown previously (Shen *et al*., 1997; van Steensel and de Lange, 1997). However, infiltrating carcinoma cells displayed much weaker staining with the Pin2/TRF1 antibodies (Figure 7b). To ensure that these signals indeed represent Pin2/TRF1, the Pin2/TRF1-specific antibodies were depleted using GST-Pin2 beads prior to immunostaining. The Pin2/TRF1-depleted antibodies showed no immunoreactivity (data not shown), confirming the specificity of the antibodies, as described (Shen *et al*., 1997).

To confirm the immunostaining results, fresh normal or tumor breast tissues were ground in liquid nitrogen and lysates were directly subjected to immunoblotting analysis, followed by semi-quantification of protein levels using ImageQuant, as described (Lu *et al*., 1999a). As an internal control, we used actin, with the Pin2/TRF1 level in each sample being expressed as a ratio between Pin2/TRF1 and actin. Out of 10 normal and 51 primary human breast cancer tissues examined, we observed that levels of Pin2/TRF1 protein in all neoplastic breast tissues were significantly lower that those present in normal control tissues (Figure 7c, Table 3). Together, both immunostaining and immunoblotting analyses indicate that expression of *PIN2/TRF1* is significantly down-regulated in most breast cancer samples examined.

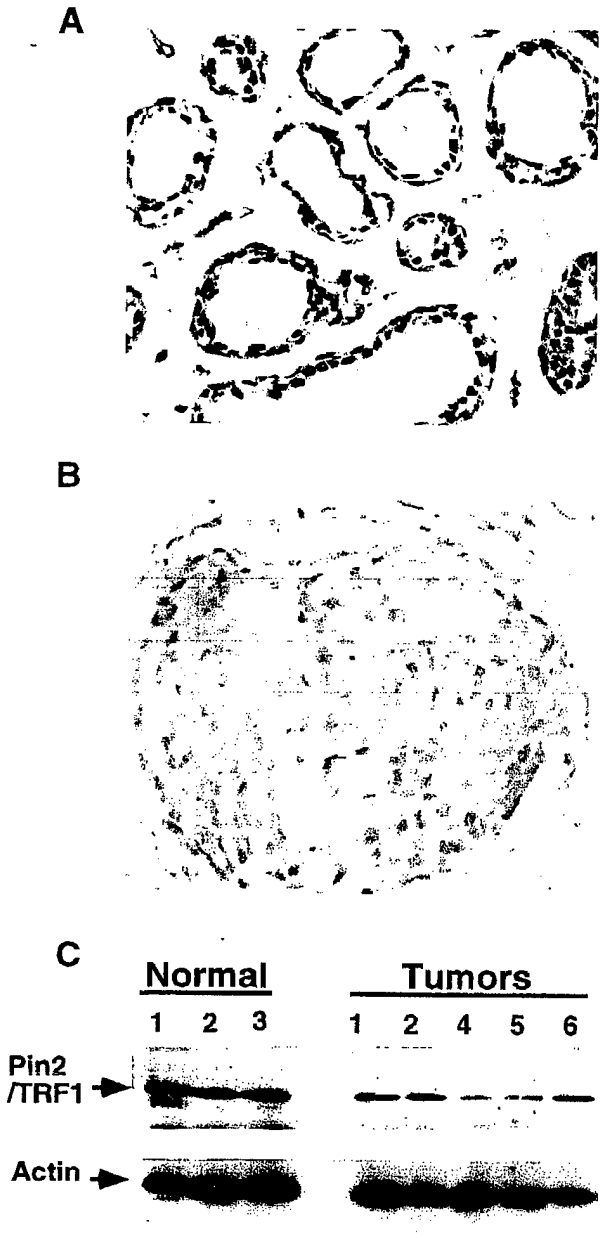

Figure 7 Down-regulation of Pin2/TRF1 expression in human breast cancer samples. (**a**, **b**) Immunostaining of Pin2/TRF1 in normal (**a**) and cancerous (**b**) human breast tissues. Fixed breast cancer sections were subjected to immunostaining with affinity-purified anti-Pin2/TRF1 antibodies. Magnification: 40×. (**c**) Comparison of Pin2/TRF1 levels in selected normal and cancerous human breast tissues. Breast tissues obtained from three normal and six breast cancer patients were powderized and the same amounts of total protein were directly separated on SDS-containing gels and transferred to membranes. The membranes were cut into two pieces and subjected to immunoblotting analysis using antibodies against to Pin2/TRF1 and actin, respectively

Discussion

We have demonstrated that up-regulation of Pin2 function results in mitotic entry and then apoptosis. Interestingly, this phenotype depends on the concentration of unbound Pin2 and on telomere length in the cells. Furthermore, overexpression of Pin2 leads to activation of caspase-3 and inhibition of caspase-3 significantly suppresses the ability of Pin2 to induce apoptosis. These results indicate that overexpression of Pin2 specifically induces mitotic entry and apoptosis

Table 3 Comparison of Pin2/TRF1 levels in normal and neoplastic breast tissues

	Number of cases	*Age range*	*Pin2/TRF1 levels* ($X \pm SD$)	p *value*
Normals	10	22–91	2.517 ± 1.635	0.0004
Tumors	51	28–90	0.835 ± 0.565	

The patient cohort included 47 invasive breast carcinoma and four ductal carcinoma *in situ*. Levels of Pin2/TRF1 in tissues were determined by immunoblotting analysis and semi-quantified using ImageQuant software, with the results being expressed as a ratio between Pin2/TRF1 and actin in each tissue. The significance of the differences in Pin2/TRF1 levels between normal controls and tumors was analysed using the Kruskall–Wallis test

likely via a mechanism that is related to telomere dysfunction. Finally, we found that levels of Pin2/TRF1 are significantly reduced in human breast cancer tissues, as compared with those in the normal control. Together with that down-regulation of Pin2/TRF1 allows cells to maintain long telomeres, these results suggest that down-regulation of Pin2/TRF1 may be important for the growth of cancer cells.

Several results support that the effects of exogenously expressed Pin2 are specific and related to telomeres shortening. First, Pin2-induced apoptosis depends on the concentration of unbound Pin2, which is likely to be higher in cells with short telomeres. Second, Pin2 induces apoptosis only in cells containing short telomeres, but not in cells containing long telomeres, similar to apoptosis induced by inhibition of telomerase (Hahn *et al*., 1999; Herbert *et al*., 1999; Zhang *et al*., 1999). Third, up-regulation of Pin2 leads to activation of caspase-3, a key executioner of apoptosis, that is responsible either partially or totally for the proteolytic cleavage of many essential proteins during apoptosis (Cryns and Yuan, 1998). Fourth, inhibition of caspase-3 suppresses the ability of Pin2 to induce apoptosis, indicating that activation of caspase-3 plays an important role during Pin2-induced apoptosis. These results argue that the ratio of 'free' and 'bound' Pin2 is crucial for cells, and increases as telomeres shorten, eventually leading to apoptotic cell death by mitotic catastrophe.

The findings that Pin2/TRF1 induces entry into mitosis and apoptosis are consistent with previous reports. Deletion of telomeres also induces mitotic arrest and apoptosis in *Drosophila* eyes *in vivo* (Ahmad and Golic, 1999). Furthermore, apoptosis is also triggered by inhibiting telomerase via expression of antisense nucleotide or dominant-negative mutants (Fu *et al*., 1999; Hahn *et al*., 1999; Herbert *et al*., 1999; Kondo *et al*., 1998a,b,c; Lee *et al*., 1998; Zhang *et al*., 1999) and inhibition of the telomeric protein TRF2 (Karlseder *et al*., 1999). These results consistently show that modulating telomere length can affect mitosis and lead to apoptosis. Furthermore, we have previously shown that the expression level of Pin2/TRF1 is cell cycle-dependent (Shen *et al*., 1997). Pin2/TRF1 is significantly increased when cells reach late G2 and M phase of the cell cycle, followed by degradation before cells exit from mitosis. Together with the findings that Pin2 contains a D-like motif similar to the destruction box present in many mitotic proteins, we have previously proposed that degradation of Pin2/TRF1 may be required for cells to exit from mitosis (Shen *et al*., 1997). Our current results, showing that overexpression of Pin2 leads to mitotic entry followed by apoptosis, further support the idea that the function of Pin2/TRF1 is tightly regulated during mitosis.

Pin2-induced apoptosis depends on telomere length in cells. Whereas Pin2 potently induces apoptosis in cells containing short telomeres, such as HeLa cells, A-T22IJE-T and A431, Pin2 fails to induce apoptosis in cells with long telomeres, such as 293, HT1080 and HeLa1.2.11, a HeLa subclone containing long telomeres, even though the protein is expressed and highly concentrated at long telomeres in these cells. This finding that the ability of Pin2 to induce apoptosis depends on telomere length may provide an explanation for why TRF1 has not been shown to induce apoptosis in some cells, including HT1080 cells (Karlseder *et al*., 1999; van Steensel and de Lange, 1997). It is also consistent with the recent demonstration that the ability of inhibiting telomerase to induce apoptosis highly depends on the length of telomeres (Zhang *et al*., 1999). Expression of dominant-negative telomerase mutants induces apoptosis only in cells that contain short telomeres, although it does not induce further shortening of telomeres (Zhang *et al*., 1999). Similarly, expression of Pin2 in those cells containing short telomeres does not further shorten telomeres (data not shown). Since telomere length is sensed by the concentration of bound telomeric proteins, as shown in the case of Rap1p (Marcand *et al*., 1997), a high concentration of bound Pin2/TRF1 in long telomere cells could be a signal that the telomeres are long enough for cells to continue dividing. Conversely, a high concentration of unbound Pin2/TRF1 in short telomere cells could indicate that the telomeres are too short for the cell to divide. This latter possibility is supported by our findings that the point mutant in the DNA-binding domain does not bind to the telomeric DNA but still potently induces apoptosis. Therefore, telomere length and the concentration of unbound Pin2 may be important signals for cell proliferation.

Although modulating the function of telomerase or either telomeric protein Pin2/TRF1 or TRF2 can all lead to apoptosis, the molecular pathways involved seem quite different. It has been demonstrated that apoptosis induced by inhibition of TRF2 is ATM- and p53-dependent (Karlseder *et al*., 1999). However, p53 is also functionally absent from all of cell lines sensitive to Pin2/TRF1-induced apoptosis in our studies due to the presence of HPV E6 or SV40 T antigen in these cells. Together with findings that Pin2 or TRF1 also potently induced apoptosis in ATM-negative A-T22IJE-T cells, these results indicate that Pin2/TRF1 induced apoptosis is ATM- and p53-independent. Similarly, p53 is also not required for apoptosis observed in telomerase-inhibited cells (Zhang *et al*., 1999). Given that both overexpression of Pin2/TRF1 and inhibition of telomerase inhibit telomere elongation (Hahn *et al*., 1999; Herbert *et al*., 1999; van Steensel and de Lange, 1997; Zhang *et al*., 1999), it is conceivable that Pin2/TRF1 may induce apoptosis via a mechanism similar to that of telomerase inhibition, although the actual signal to activate apoptosis remains to be elucidated. At least two different signaling pathways exist in telomere-mediated apoptosis. One is the ATM- and p53-dependent pathway that is activated by inhibition of TRF2 and the other is the ATM- and p53-independent pathway that is activated by inhibition of telomere elongation via up-regulating Pin2/TRF1 or inhibiting telomerase. The ability to pinpoint the induction of apoptosis in these two pathways may provide a powerful tool to investigate

the molecular nature of the apoptotic response to telomere dysfunction.

Although it remains to be determined whether Pin2/TRF1 is able to induce mitosis and apoptosis under physiological conditions, there are at least two pathological conditions where Pin2/TRF1-induced apoptosis may be important. One condition is the genetic disorder ataxia-telangiectasia caused by ATM mutations. These patients are hypersensitive to irradiation and cells derived from the patients contain short telomeres and display a prominent G2/M checkpoint defect upon irradiation. These ATM-negative cells fail to delay entry into mitosis and instead are prone to enter mitosis and apoptosis after irradiation (Metcalfe *et al.*, 1996; Pandita *et al.*, 1995; Smilenov *et al.*, 1997; Xia *et al.*, 1996). Significantly, the hypersensitivity to ionizing radiation is correlated with telomere loss (Metcalfe *et al.*, 1996; Pandita *et al.*, 1995; Smilenov *et al.*, 1997; Xia *et al.*, 1996). Interestingly, we have shown that ATM binds and negatively regulate the function of Pin2/TRF1 presumably via phosphorylation (Kishi *et al*, a manuscript submitted). More significantly, if the function of endogenous Pin2/TRF1 in ATM-negative cells is inhibited by stably expressing dominant-negative Pin2, cells are no longer sensitive to irradiation. Following irradiation, the Pin2/TRF1-inhibited A-T cells do not enter mitosis and apoptosis, but instead delay entry into mitosis, which is a normal DNA damage response for repairing damaged DNA. These results indicate that inhibition of endogenous Pin2/TRF1 function is sufficient to prevent DNA damage-induced mitosis and apoptosis, and also suggest that endogenous Pin2/TRF1 in ATM-negative cells is able to induce mitosis and apoptosis, at least upon DNA damage (Kishi *et al.*, manuscript submitted). Another pathological condition where Pin2/TRF1-induced apoptosis may be significant is cancer cells. In contrast to most somatic cells, where telomeres are shortened with each cell division and there is a limited life span, cancer cells have an unlimited cell division potential and have to maintain their telomeres (Greider and Blackburn, 1996; Lundblad, 2000; Zakian, 1995). To maintain this continuous cell division, the function of Pin2/TRF1 is likely to be down-regulated in these cancer cells since up-regulation of Pin2/TRF1 results in telomere shortening, as shown previously (van Steensel and de Lange, 1997), and induces apoptosis, as shown here. Indeed, we have now found that Pin2/TRF1 is significantly down-regulated in most human breast cancer samples, as confirmed both by immunostaining and immunoblotting analysis. A recent immunohistochemical study also revealed a similar down-regulation of Pin2/TRF1 in gastrointestinal tumors (Aragona *et al.*, 2000). Although the relationship between down-regulation of Pin2 and telomere length in tumor cells remains to be addressed, these results suggest that down-regulation of Pin2/TRF1 may be a general phenomenon in cancer and this down-regulation may allow cancer cells to extend their proliferative potential. Further studies on the role of Pin2/TRF1 in modulating cell proliferation and cell death may help understand the role of telomere maintenance in cellular aging and transformation.

Materials and methods

Transient transfection and apoptosis assays

For detecting apoptosis using β-gal assay, cells were co-transfected with pSV2-lacZ and vector encoding wild-type or mutant Pin2 for 48–60 h by using the Superfect reagents (Qiagen), fixed with 0.5% glutaraldehyde and stained with X-gal, as described (Kumar *et al.*, 1994; Mayo *et al.*, 1997). For the TUNEL assay, cells were cotransfected with Pin2 expression construct and the cell surface marker CD20 for 36 h and then stained with anti-CD20 antibody (Pharmingen), as described (Zhu *et al.*, 1993). The stained cells were subjected to TUNEL staining and analysed by flow cytometry, as described (Douglas *et al.*, 1998; Gavrieli *et al.*, 1992). To directly observe the morphology of Pin2 expressing cells, Pin2 and its various mutants were expressed as C-terminal fusion proteins with GFP in cells (Clontech). Respective vectors were used in all transfections as controls. Transfected living cells were monitored over time and fixed at various time points. The indexes of interphase, mitotic and apoptotic cells were determined after staining the cells with the DNA-binding dye DAPI or the mitosis-specific monoclonal antibody MPM-2, as described (Lu and Hunter, 1995; Shen *et al.*, 1997). The apoptosis rate was determined by counting about 300–400 GFP-positive cells.

Analysis of telomere restriction fragment length

Telomere restriction fragment length was determined, as described previously (van Steensel and de Lange, 1997). In brief, genomic DNA was isolated from the cultured cells using QIAamp Tissue Kit (QIAGEN), and digested with *Hinf*I and *RSA*I (New England Bio Labs) to generate the telomere restriction enzyme fragments. Ten μg of genomic DNA was separated on a 0.7% agarose gel. This gel was hybridized directly to a ^{32}P-labeled telomere probe, which was made with (AATCCC) primer using pSP73 StyI1 plasmid as a template in Klenow fragment.

Cell cycle analysis

To enrich cells in G1, cells were treated with 20 μM lovastatin for 20 h, as described (Jakobisiak *et al.*, 1991; Keyomarsi *et al.*, 1991). To block cells at mitosis, cells were incubated 100 ng/ml nocodazole for 16 h. For cell cycle analysis, cells were harvested by trypsinization, re-suspended in DMEM supplemented with 10% serum, washed in PBS, and then fixed in 70% ethanol. After washing cells once with PBS containing 1% BSA, DNA was stained with propidium iodide (10 μg/ml) containing 250 μg/ml of ribonuclease A, followed by flow cytometry analysis (Becton-Dickinson), as described (Lu and Hunter, 1995).

Flow cytometric analysis of caspase-3 activation and treatment of caspase-3 inhibitor

After 28 h of transfection with GFP-Pin2 or TM-GFP expression construct, HeLa cells were fixed and then immunostained with cleaved caspase-3 antibody (Cell Signaling Technology), followed by Rhodamine-conjugated anti-rabbit secondary antibodies, as described (Belloc *et al.*, 2000).

The stained cells were analysed by flow cytometry for the detection of caspase-3 activation. To inhibit caspase-3, 10 μM Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) was added to cells before transfection.

Expression of Pin2/TRF1 in human cancer tissues

Fifty-one cancerous and 10 normal breast tissue specimen were randomly selected. Tissue from the core of the tumor had been snap frozen in liquid nitrogen and powderized using a Microdismembrator (Braun). About 10 μg of the powderized tissues were re-suspended in 100 μl of SDS sample buffer. Immunoblotting analysis with anti-Pin2/TRF1 and anti-actin antibodies was performed as described (Shen *et al.*, 1997). Levels of Pin2/TRF1 were semi-quantified using Imagequant and the significance of the differences in Pin2/TRF1 levels between normal and cancer tissues was analysed, as described (Lu *et al.*, 1999a). To detect the localization of Pin2/TRF1 in human tissues, 50 μm sections were cut from breast cancer tissues, and then microwaved in an antigen retrieval buffer (Biogenex), as described by the manufacturer. Endogenous peroxidase activity was blocked with H_2O_2, the sections were incubated with anti-Pin2/TRF1 antibodies that had been purified using GST-Pin2 glutathione beads (Shen *et al.*, 1997), and visualized by the immunoperoxidase staining protocol, as described (Lu *et al.*, 1999a).

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Prolyl isomerase Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC [au: please cut to 3 lines]

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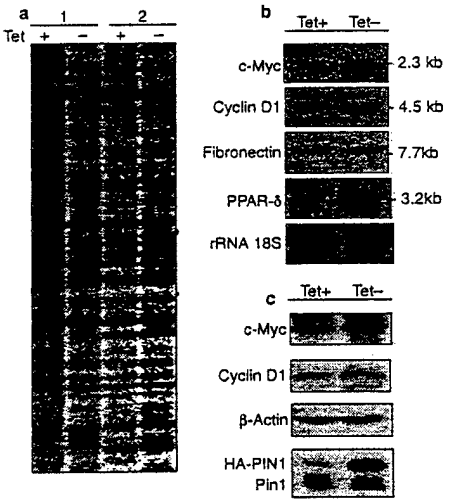
Phosphorylation on a serine or threonine residue preceding proline (Ser/Thr-Pro) is a key regulatory mechanism, and the conformation of certain phosphorylated Ser/Thr-Pro bonds is regulated specifically by the prolyl isomerase Pin1. Whereas the inhibition of Pin1 induces apoptosis, Pin1 is strikingly overexpressed in a subset of human tumours. Here we show that Pin1 regulates β-catenin turnover and subcellular localization by interfering with its interaction with adenomatous polyposis coli protein (APC) [O.K.?]. A differential-display screen reveals that Pin1 increases the transcription of several β-catenin target genes, including those encoding cyclin D1 and c-Myc. Manipulation of Pin1 levels affects the stability of β-catenin *in vitro*. Furthermore, β-catenin levels are decreased in Pin1-deficient mice but are increased and correlated with Pin1 overexpression in human breast cancer. Pin1 directly binds a phosphorylated Ser-Pro motif next to the APC-binding site in β-catenin, inhibits its interaction with APC and increases its translocation into the nucleus. Thus, Pin1 is a novel regulator of β-catenin signalling and its overexpression might contribute to the upregulation of β-catenin in tumours such as breast cancer, in which APC or β-catenin mutations are not common.

Upregulation of the oncogenic transcriptional activator β-catenin has a pivotal role in the development of cancer[1–3]. One of the key β-catenin regulators is adenomatous polyposis coli protein (APC), [O.K.?] which is encoded by the tumour-suppressor gene that is mutated in familial adenomatous polyposis coli[4–9]. The tumour-suppressing activity of APC largely involves controlling the nuclear accumulation of the oncogenic transcriptional activator β-catenin[4–10]. The stable overexpression of β-catenin caused by mutations in APC or β-catenin leads to an accumulation of β-catenin in the nucleus, where it induces a set of genes critical for the development of cell transformation and cancer, including those encldong cyclin D1, c-Myc, fibronectin and peroxisome-proliferator-activated receptor-δ (PPAR-δ)[11–16]. There are two major mechanisms by which APC modulates the concentration of β-catenin in the nucleus. First, APC binds and assembles β-catenin into a multiple protein complex, including glycogen synthase kinase-3β (GSK-3β) and trigger the degradation of β-catenin[17,18]. However, the activation of Wnt signalling inhibits the phosphorylation of β-catenin by GSK-3β, resulting in the stabilization of β-catenin in the cytoplasm and nucleus[1–3,17,19]. Second, APC binds the nuclear β-catenin and exports it to the cytoplasm for degradation[20–22]. Thus, a crucial step in the APC-mediated regulation of β-catenin is the interaction between them. Indeed, this interaction is often disrupted in many mutations of APC in cancer[5,6]. Therefore, disruption of the interaction between APC and β-catenin has a pivotal role in oncogenesis. However, it is not known whether this interaction can be regulated.

Although genetic mutations of APC or β-catenin are often found in some tumours, such as colon cancer[1–3], they are rarely observed in others such as breast cancer[23–25]. However, compelling evidence has indicated a crucial role for signalling by β-catenin in the tumorigenesis of breast cancer[23–26]. Furthermore, β-catenin levels are significantly upregulated and are correlated with poor prog-



Figure^1 **Activation of genes downstream of those encoding β-catenin/TCF by overexpression of Pin1. a**, Representative results of the differential display screen. Pin1 was induced in the breast-cancer cell line MCF-7 with the Tet-Off gene expression system (Clontech). MCF-7 cells were cultured in the presence (Tet+) or absence (Tet–) of tetracycline for 24^h and the isolated RNA was subjected to a differential display screen. The results obtained from the two respective combinations of extended primers are shown; the bands were excised for further analysis are marked with arrowheads: A, upregulated CD97; B, upregulated PPAR-δ; C, a downregulated unknown gene. b,^c, Total RNA and cell lysates were prepared from the same cells as described in a and subjected to northern blotting (b) or immunoblotting analysis (c).

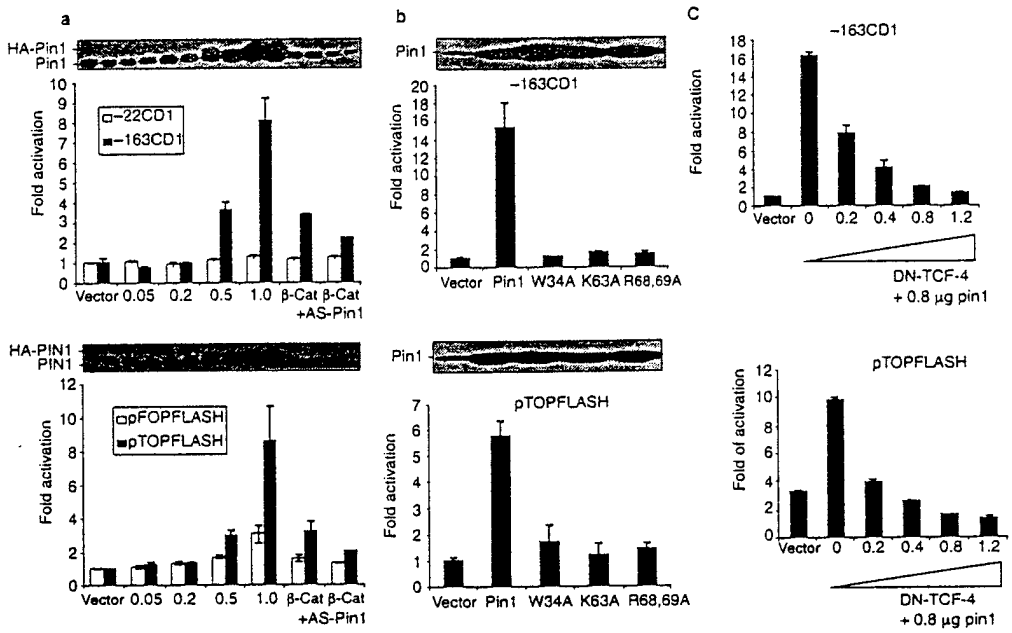


Figure 2 **Pin1 transactivates genes downstream of those encoding β-catenin/TCF through TCF sites. a**, HeLa cells were transfected with different concentrations of Pin1 and various reporter constructs as indicated, followed by an assay of the luciferase activity. pRL-TK *Renilla* luciferase reporter construct was co-transfected in each sample to normalize for transfection efficiency. The activity of the reporter luciferase was expressed relative to the activity in control-vector-transfected cells, which is defined as 1.0. –22CD1, the cyclin D1 minimal promoter region from –22 to +14; –163CD1, the cyclin D1 minimal promoter region from –163 to +14 containing three TCF-binding sites; pTOPFLASH, three copies of the optimal TCF motif CCTTTGATC; pFOPFLASH, [**'FOPFLAS'?**] three copies of the mutant motif CCTTTGGCC. Expression of endogenous Pin1 and exogenous haemagglutinin (HA)labelled Pin1 was detected by western blot analysis with a monoclonal antibody against Pin1; this is shown at the top of the panels. All experiments were repeated in triplicate and are expressed as means ± s.d. b, HeLa cells were co-transfected with Pin1, and its WW-domain mutant (W34A) or its PPIase-domain mutants (K63A and R68,69A) with –163CD1 or pTOPFLASH luciferase reporter construct, **[AUTHOR: O.K.?—what was transfected with what?]** followed by the luciferase assay. c, HeLa cells were co-transfected with Pin1, –163CD1 or pTOPFLASH luciferase reporter construct and increasing concentrations of dominant-negative TCF-4 (DN-TCF-4), followed by the luciferase assay.

nosis, acting as a strong and independent prognostic factor in human breast-cancer patients[24]. Additional mechanisms can therefore be used to upregulate β-catenin levels in breast cancer. We have recently shown that Pin1 is strikingly overexpressed in human breast cancer and some other tumours[27]. Furthermore, Pin1 levels are correlated with the tumour grade and with cyclin D1 levels in breast-cancer tissues[27]. These results indicate that Pin1 might have a role in oncogenesis.

Pin1 is a peptidyl-prolyl *cis–trans* isomerase (PPIase) [AUTHOR: O.K.?] that isomerizes only phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) peptide bonds[28–31]. Pin1-catalysed isomerization regulates the conformation of a subset of phosphoproteins such as Cdc25C and the microtubule-associated protein tau, thereby affecting their activity and/or protein–protein interactions[30–35]. Interestingly, the depletion of Pin1 in tumour cells induces apoptosis[28,36] and also contributes to neuronal death in Alzheimer's disease[34]. Conversely, Pin1 is overexpressed in many human tumours such as breast and prostate cancer, and increases the transcriptional activity of c-Jun towards the cyclin D1 promoter[27]. These results indicate that Pin1 overexpression might promote tumour cell growth by altering gene expression.

To test this hypothesis, we here used a differential display screen and found that Pin1 transactivated several β-catenin target genes. Furthermore, Pin1 increased the stability of β-catenin in cells, which is substantiated by the findings that β-catenin was increased and correlated with Pin1 in breast cancer tissues, but drastically decreased in Pin1-deficient mouse tissues. Moreover, Pin1 stabilized and subsequently increased the nuclear fraction of β-catenin by preventing its interaction with APC. These findings uncover a novel mechanism for regulating signalling by β-catenin and support a role for Pin1 in tumorigenesis.

Results

Pin1 enhances the expression of genes downstream of those encoding β-catenin/T-cell factor (TCF). To examine the role of Pin1 overexpression in human breast cancer, we used a newly developed differential display method[37,38] to identify genes and signalling pathways regulated by Pin1. By comparing gene expression patterns of about 10,000 complementary DNA fragments between Pin1-overexpressing breast-cancer MCF-7 cells and control MCF-7 cells (see Fig. 1a), we selected and sequenced 48 cDNA fragments that were obviously expressed differentially between Pin1-induced and non-induced conditions. From these 48 clones we reproducibly identified 17 known genes whose expression was upregulated or downregulated by the overexpression of Pin1 (Table 1). Interestingly, a database search revealed that four of the twelve upregulated genes identified were downstream of those encoding β-catenin/TCF, which included those for cyclin D1, c-Myc, PPAR-δ and fibronectin[13–16]. To confirm that these genes are indeed induced by Pin1, we isolated messenger RNAs and subjected them to northern blotting analysis. Figure 1b shows that mRNA levels of these genes were indeed higher in cells expressing Pin1 than those in control cells. Furthermore, immunoblotting analysis with cell lysates

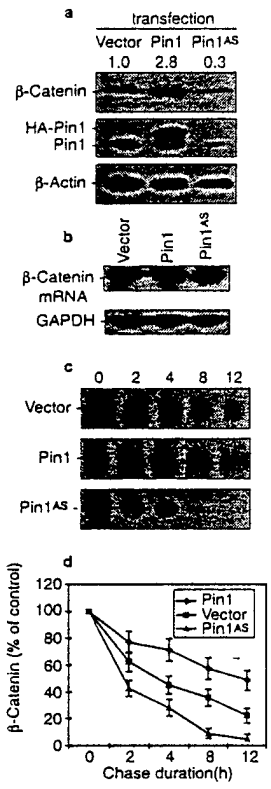


Figure 3 **Pin1 stabilizes cellular β-catenin. a**, HeLa cells were transfected with 0.5 μg of a sense (Pin1) or antisense Pin1 ($Pin1^{AS}$) expression construct or control vector and harvested 48 h after transfection, followed by immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-β-actin antibodies. Numbers above the gel image indicate the fold induction of endogenous β-catenin normalized with β-actin. **b**, Total RNA was extracted from the same cells as those in **a** and subjected to northern blot analysis. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. **c**, **d**, Subconfluent cells were transfected with a sense (Pin1) or antisense Pin1 ($Pin1^{AS}$) expression construct or control vector. After 24 h, cells were pulse-labelled with [^{35}S]methionine for 1 h and chased for the durations indicated (**c**). Labelled β-catenin was detected by immunoprecipitation with anti-β-catenin, followed by SDS–PAGE and autoradiography. The radioactivity of immunoprecipitated β-catenin was quantified with a PhosphorImager and normalized to the 0 h point (**d**). Results shown are means ± s.d. **[AUTHOR: O.K.?]** for three independent experiments.

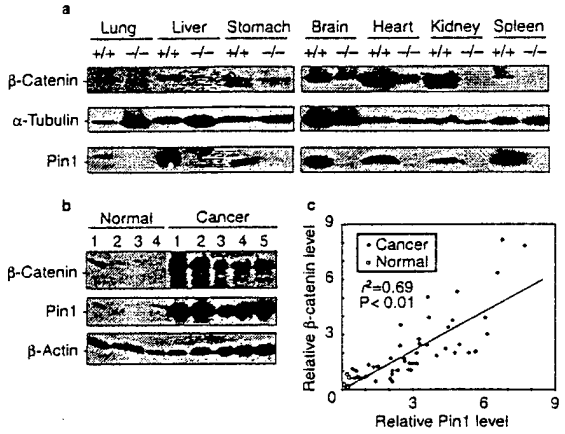


Figure 4 **β-Catenin levels are decreased in Pin1-deficient mice but increased in breast-cancer samples overexpressing Pin1. a**, Different issues of wild-type (+/+) or Pin1 knockout mice (−/−) were homogenized and then subjected to immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-α-tubulin antibodies. **b**, Normal breast and breast-cancer tissues were powderized **[AUTHOR: please explain this term—do you mean 'powdered', 'extracted with acetone and dried', or something else?]** and subjected to immunoblotting analysis with anti-β-catenin, anti-Pin1 or anti-α-tubulin antibodies. Normal 1–4, non-neoplastic breast; cancer 1 and 2, Bloom and Richardson grade III; cancer 3–5: grade II. **c**, The correlation between Pin1 and β-catenin protein levels in malignant and normal breast tissues. Immunoblot analysis was performed on 45 breast-cancer and 6 normal tissue samples, as in **b**. The densities of bands were quantified by NIH Image, normalized with β-actin and plotted. The correlation was tested by a linear regression analysis ($r^2 = 0.69$, $P < 0.01$).

from the same cells also showed an enhanced expression of c-Myc and cyclin D1 proteins (Fig. 1c). These results indicate that Pin1 can affect the gene expression pattern by activating the β-catenin/TCF signalling pathway.

To confirm that Pin1 activates the β-catenin/TCF signalling pathway, we used two sets of reporter constructs in a β-catenin/TCF reporter gene assay, as described previously[7,15]. Although Pin1 cDNA had no significant effect on the −163CD1 promoter at low concentrations, as shown previously[27], Pin1 increased the activity of both the −163CD1 and pTOPFLASH promoters in a dose-dependent manner. In contrast, Pin1 had no significant effect on the control promoter, that is, on either the −22CD1 promoter or the pFOPFLASH [AUTHOR: 'FOPFLAS' in Methods— which is correct?] promoter (Fig. 2a). Furthermore, the depletion of endogenous Pin1 by the expression of an antisense Pin1 construct [AUTHOR: O.K.?] inhibited the ability of β-catenin to activate the −163CD1 or pTOPFLASH promoter (Fig. 2a). These results demonstrate that Pin1 increases the activity of both the cyclin D1 and pTOPFLASH promoters.

Both the binding and isomerizing activities of Pin1 are normally required for Pin1 to regulate the function of its substrates[29,30,32–34]. To examine whether any one or both of these activities are required for Pin1 to modulate the activity of the −163CD1 or pTOPFLASH promoter, we used Pin1 mutants, $Pin1^{W34A}$, $Pin1^{K63A}$ and $Pin1^{R68,69A}$, which fail to bind phosphoproteins or isomerize pSer/Thr-Pro bonds, respectively[31–33]. [AUTHOR: please clarify what you mean by 'respectively'—three mutants but only two outcomes] As shown in Fig. 2b, in contrast to wild-type Pin1, neither the WW-domain mutant ($Pin1^{W34A}$) nor the PPIase mutants ($Pin1^{K63A}$ and $Pin1^{R68,69A}$) increased the activity of the −163CD1 or pTOPFLASH promoter. These results indicate that both phosphoprotein-binding and phosphorylation-specific isomerase activities of Pin1 are required for activation of the β-catenin-dependent transcription.

To examine whether these effects of Pin1 on the activity of the cyclin D1 and pTOPFLASH promoters are mediated by the TCF-binding site, we used truncated TCF-4 (DN-TCF4), which has been shown to act as a dominant-negative mutant[7,13]. When co-transfected into cells with Pin1, the dominant-negative TCF-4 mutant decreased the ability of Pin1 to increase the −163CD1 or pTOPFLASH promoter activity in a dose-dependent manner (Fig. 2c). These results indicate that Pin1 enhances the ability of the β-catenin/TCF signalling pathway to activate its downstream target genes through the TCF sites, which is consistent with the findings that Pin1 did not activate the pFOPFLASH [AUTHOR: ?correct] and −22CD1 promoters.

Pin1 stabilizes β-catenin by post-translational regulation. The fact

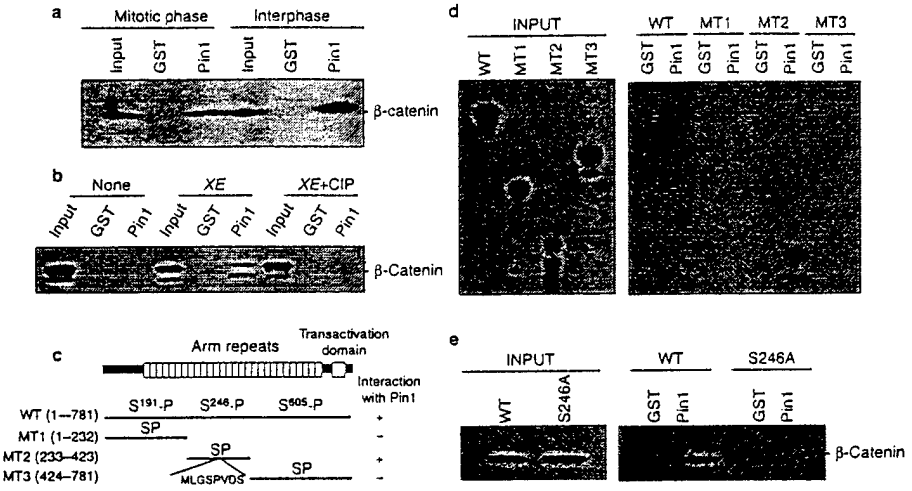


Figure 5 **Pin1 binds β-catenin phosphorylated on the Ser 246-Pro motif in the middle of the Armadillo repeat domain. a**, Glutathione–agarose beads containing GST or GST–Pin1 were incubated with extracts of HeLa cells in interphase or undergoing mitosis. After washing, binding proteins were subjected to immunoblotting analysis with anti-β-catenin antibodies. **b**, β-Catenin protein was synthesized by transcription and translation *in vitro* in the presence of [^{35}S]methionine. The labelled protein was incubated with control buffer (None), *Xenopus* extracts (*XE*) or *Xenopus* extracts followed by treatment with calf intestinal alkaline phosphatase (*XE*+CIP). These proteins were separated on SDS-containing gels either directly (input) or after GST pulldown experiments with glutathione beads containing GST or GST–Pin1. **c**, Schematic representation of wild-type (WT) β-catenin and its truncation mutants. **d**, Wild-type β-catenin and its truncation mutants were labelled with [^{35}S]methionine, incubated with *Xenopus* extracts and then subjected to GST pulldown experiments. **e**, β-Catenin and its site-directed S246A mutant were labelled with [^{35}S]methionine, incubated with *Xenopus* extracts and then subjected to GST pulldown experiments.

that Pin1 increases the transcription of the genes downstream of those encoding β-catenin/TCF indicates that Pin1 might affect the protein levels of β-catenin. To examine this possibility, we examined the effect of manipulating cellular Pin1 levels on levels of β-catenin protein and mRNA. Figure 3a shows that the overexpression and depletion of Pin1 significantly increased and decreased β-catenin protein levels, respectively. However, no difference in β-catenin mRNA level was observed when Pin1 was overexpressed or depleted (Fig. 3b), indicating that Pin1 does not increase the transcription of the gene encoding β-catenin. To investigate whether these effects of Pin1 on the β-catenin protein levels were due to post-translational regulation, we used a pulse–chase study to analyse the effects of Pin1 on the stabilization of endogenous β-catenin. The metabolic stability of β-catenin was significantly increased in Pin1-overexpressed cells but decreased in Pin1-depleted cells (Fig. 3c). Quantification by PhosphorImager revealed that, in comparison with vector-transfected control cells, the half-life of β-catenin was significantly affected by manipulating cellular concentrations of Pin1, especially at the early time points (Fig. 3d). Taken together, these results indicate that Pin1 increases the levels of β-catenin protein by inhibiting its degradation.

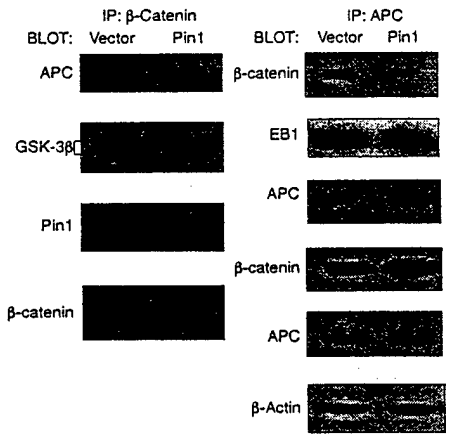
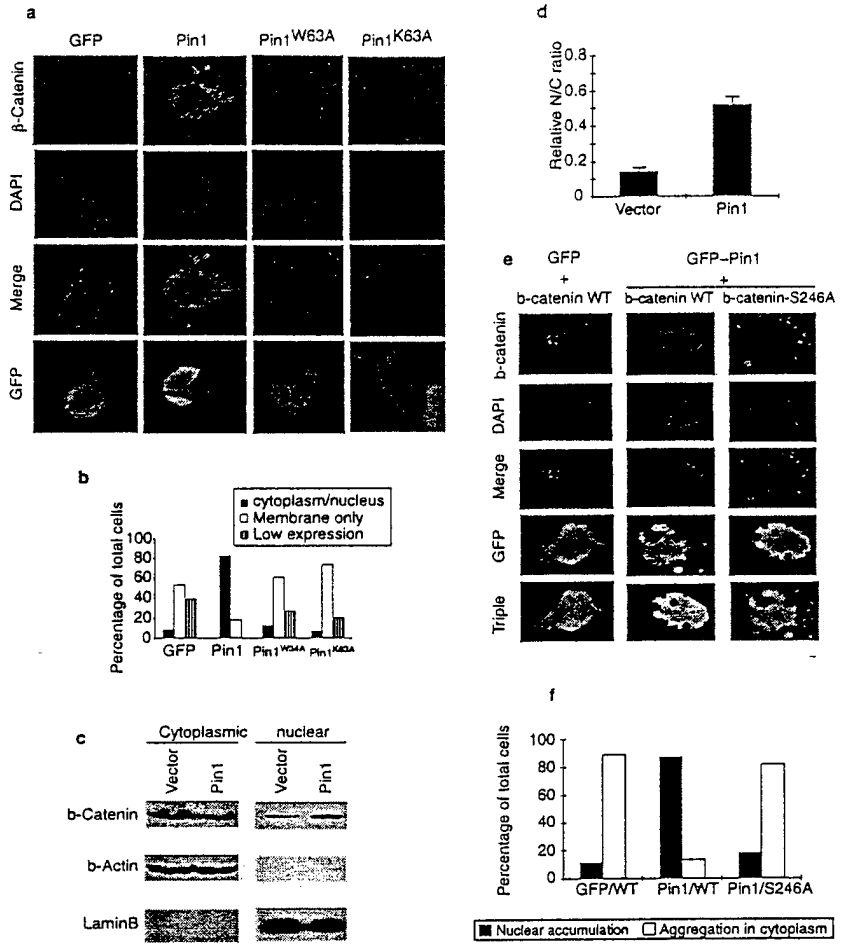


Figure 6 **Pin1 selectively inhibits the interaction between β-catenin and APC.** HeLa cells were transfected with Pin1 expression construct or control vector for 24 h, and lysates were subjected to immunoprecipitation (IP) with anti-β-catenin (a) or anti-APC antibody (b). Immunoprecipitates were subjected to SDS–PAGE and immunoblotting analysis with various antibodies as indicated. Experiments were repeated several times with similar results.

The level of β-catenin is downregulated in Pin1 knockout mice but upregulated and correlated with Pin1 overexpression in human breast cancer tissues. To determine the effects of Pin1 on β-catenin levels *in vivo*, we first examined β-catenin protein levels in tissues of Pin1 knockout mice and in human breast-cancer samples. Pin1 knockout mice have been generated previously and shown to develop normally[39]. As expected, Pin1 was not detected in Pin1 knockout mouse tissues (Fig. 4a). Importantly, amounts of β-catenin protein were decreased significantly in all tissues examined from Pin1 knockout mice, in comparison with those from wild-type mice (Fig. 4a), indicating that the β-catenin level is downregulated in Pin1 knockout mice. This is consistent with our new findings that mice lacking Pin1 demonstrate phenotypes remarkably similar to those in cyclin D1 knockout mice (Y.-C.L., [AUTHOR: please give all names (with initials)], unpublished results).

We have recently shown that Pin1 is overexpressed in human breast-cancer tissues and its expression level is correlated with tumour grade and cyclin D1 levels[27]. To examine whether Pin1 overexpression is correlated with β-catenin levels in breast-tumour tissues, we determined levels of Pin1 and β-catenin in breast-cancer tissues. Figure 4b shows that both β-catenin and Pin1 were highly overexpressed in breast-cancer tissues in comparison with



normal tissues. We performed the same immunoblot analysis and control GST, specifically precipitated β-catenin from both inter-

Figure 7 **Pin1 induces nuclear translation of β-catenin. a, b,** HeLa cells were transfected for 24 h with the construct expressing GFP, GFP–Pin1, its WW domain mutant ($Pin1^{W34A}$) or its PPIase domain mutant ($Pin1^{K63A}$). Cells were fixed and stained with anti-β-catenin antibodies and DAPI to detect endogenous β-catenin and DNA, respectively (**a**). Localization and expression of β-catenin were scored in 100 transfected cells (**b**). **c, d,** HeLa cells were transfected for 24 h with Pin1 expression construct or the control vector. Cells were fractionated in hypotonic buffer into the nuclear and cytoplasmic fractions, followed by immunoblotting analysis with anti-β-catenin, anti-lamin B or anti-β-actin antibody. Lamin B and β-actin were used as nuclear and cytoplasmic markers, respectively (**c**). Relative amounts of nuclear and cytoplasmic (N/C) β-catenin were semi-quantified with ImageQuant and normalized with lamin B or β-actin (**d**). **e, f,** HeLa cells were transfected with GFP–vector and RFP–β-catenin-WT, GFP–Pin1 and RFP–β-catenin or GFP–Pin1 and RFP–β-catenin-S246A for 24 h. Cells were fixed and stained with DAPI and analysed under a fluorescence microscope (**e**). Localization of β-catenin was scored in 100 transfected cells (**f**).

quantified the expression level of both Pin1 and β-catenin in 45 breast-cancer and 6 normal breast tissues. Regression analysis revealed that the levels of β-catenin were significantly correlated with those of Pin1 in these tissues ($r^2 = 0.69$, $P < 0.01$) (Fig. 4c). These results demonstrate a close relationship between Pin1 and β-catenin levels under both physiological and pathological conditions.

Pin1 binds β-catenin phosphorylated on the Ser 246-Pro motif. To explore the molecular mechanism by which Pin1 stabilizes β-catenin, we investigated whether β-catenin is a Pin1 substrate, because β-catenin contains three potential Ser-Pro motifs. To examine the interaction between Pin1 and β-catenin *in vitro*, we used glutathione *S*-transferase (GST)-Pin1 pulldown experiments, as described previously[32,33]. As shown in Fig. 5a, GST-Pin1, but not phase and mitotic HeLa cell extracts, indicating that Pin1 binds cellular β-catenin independently of the cell cycle. Next, we examined whether Pin1 forms stable complexes with β-catenin in cells. When cells that expressed haemagglutinin-labelled Pin1 were subjected to co-immunoprecipitation experiments, Pin1 was detected in anti-β-catenin immunoprecipitates (Fig. 6a), demonstrating the interaction of these two proteins *in vivo*. These results demonstrate that Pin1 binds β-catenin both *in vitro* and *in vivo*.

We next examined whether the interaction between Pin1 and β-catenin depends on the phosphorylation of β-catenin at a specific Ser/Thr-Pro motif. Because the kinase(s) upstream of β-catenin remain to be determined, we produced ^{35}S-labelled β-catenin by transcription and translation *in vitro*, then phosphorylated it with *Xenopus* extracts, which have been shown to contain many protein

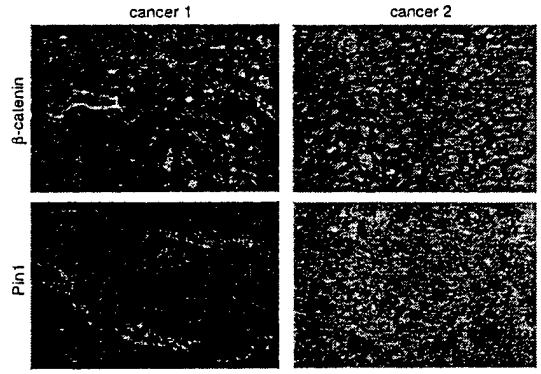


Figure 8 **Correlation between Pin1 overexpression and β-catenin localization in human breast-cancer tissues.** Breast-cancer tissues were stained with anti-β-catenin or anti-Pin1 antibody and revealed by staining with DAB. **[AUTHOR: please spell out DAB]** Left panels, a representative high-Pin1 staining with an accumulation of β-catenin in the cytoplasm and nucleus; right panels, a representative low-Pin1 staining with a localization of β-catenin at the membrane.

kinases[40]. Pin1 bound ^{35}S-β-catenin only after it had been phosphorylated by interphase or mitotic extracts from *Xenopus* (Fig. 5b, and data not shown). Furthermore, pretreatment of phosphorylated β-catenin with calf intestinal alkaline phosphatase (CIP) completely abolished the ability of Pin1 to bind β-catenin (Fig. 5b), as demonstrated previously for the Pin1–Cdc25 or Pin1–tau interaction[32,34]. These results demonstrate that the Pin1 binding depends on the phosphorylation of β-catenin.

β-Catenin contains only three Ser-Pro motifs (Fig. 5c). To determine the Pin1-binding site in β-catenin, we constructed and expressed three different β-catenin fragments, each containing one Ser-Pro motif (Fig. 5c). When these fragments were synthesized *in vitro* and phosphorylated by *Xenopus* extracts, followed by a GST pulldown assay, only the MT2 fragment (residues 233–423), not the MT1 or the MT3 fragment, bound Pin1 (Fig. 5d). Interestingly, the MT2 fragment contained the Ser 246-Pro motif, which is surrounded by hydrophobic residues similarly to the optimal binding motif selected by Pin1, as determined by screening-orientated degenerate peptide libraries[30]. To confirm the Pin1 binding site we mutated Ser 246 of β-catenin to alanine (S246A) and performed a GST pulldown assay after incubation with *Xenopus* extracts. As expected, in contrast to wild-type β-catenin, the S246A β-catenin mutant did not bind Pin1 protein (Fig. 5e). These results indicate that the Pin1-binding site is the phosphorylated Ser 246-Pro in the middle of the Armadillo repeats in β-catenin.

Pin1 blocks the interaction between β-catenin and APC. The Pin1-binding site (Ser 246-Pro) in β-catenin is very close to the APC binding site, on the basis of the crystal structure of β-catenin[41,42], indicating that Pin1 might affect the interaction of β-catenin with APC. To examine this possibility, cells were transfected with Pin1 or control vectors and then subjected to immunoprecipitation with antibodies against APC or β-catenin. As shown previously[7–9], β-catenin was detected in anti-APC immunoprecipitates and APC was detected in anti-β-catenin immunoprecipitates in control-vector-transfected cells, confirming the interaction *in vivo* between these two proteins (Fig. 6). However, in cells overexpressing Pin1, significantly less APC was detected in anti-β-catenin immunoprecipitates (Fig. 6a). Similarly, much less β-catenin was immunoprecipitated by anti-APC antibodies (Fig. 6b). Furthermore, these differences were highly specific because te overexpression of Pin1 had no detectable effect on the binding of β-catenin to GSK-3β or of APC to the APC-binding protein EB1 (Fig. 6a, b). These results demonstrate that Pin1 specifically inhibits the interaction between β-catenin and APC.

Pin1 alters the subcellular localization of β-catenin. Recent studies have shown that APC is important in controlling the localization of β-catenin by exporting it from the nucleus to the cytoplasm[20–22,43]. If Pin1 inhibits the binding of β-catenin to APC, Pin1 might affect the subcellular localization of β-catenin. We therefore investigated the subcellular localization of β-catenin. As shown previously[20,44–46], β-catenin was observed along the inner portion of the cell membrane in the cells transfected with the green fluorescent protein [O.K.?] (GFP) vector (Fig. 7a). However, in GFP–Pin1-transfected cells, β-catenin was detected mainly in the nucleus and the perinuclear portion (Fig. 7a, b). In contrast, the subcellular localization of β-catenin was unaffected by the Pin1 mutants containing a mutation either in the WW domain ($Pin1^{W34A}$) or in the PPIase domain ($Pin1^{K63A}$) (Fig. 7a, b). These results indicate that both the binding and isomerization activities of Pin1 are required to increase the nuclear fraction of β-catenin.

To confirm this Pin1-induced accumulation of nuclear β-catenin, we fractionated cells into cytoplasmic and nuclear fractions and subjected them to immunoblotting with anti-β-catenin antibodies. In comparison with vector-transfected cells, the concentration of β-catenin was slightly decreased in the cytoplasmic fraction but significantly increased in the nuclear fraction in Pin1-overexpressing cells (Fig. 7c). Semi-quantification revealed that the nuclear/cytoplasmic ratio of β-catenin was increased almost threefold in cells overexpressing Pin1 (Fig. 7d). Thus, both an immunocytochemistry analysis and a cell fractionation experiment confirmed that Pin1 facilitates the nuclear localization of β-catenin, which is consistent with its abilities to disrupt the interaction between β-catenin and APC and to induce target genes downstream of that for β-catenin.

To determine whether the mutation of Ser 246 affects the ability of Pin1 to affect the subcellular localization of β-catenin, we co-transfected HeLa cells with Pin1 and wild-type or S246A mutant β-catenin, and then examined the subcellular localization of β-catenin. Because the overexpression of exogenous β-catenin causes its spontaneous nuclear translocation and cell death[46–48], we transfected cells with a small amount of red-fluorescent-protein [O.K.?] (RFP)-conjugated β-catenin DNA. Under these conditions, RFP–β-catenin displayed a dotted-type aggregation in cytoplasm (Fig. 7e), as reported previously[46]. Importantly, β-catenin was stabilized and accumulated in the nucleus when co-transfected with GFP–Pin1, but not control GFP (Fig.7e, f). In contrast, Pin1 had no effect on the localization of the S246A mutant β-catenin (Fig.7e, f), which is consistent with the findings that this mutant was not a Pin1 substrate (Fig. 5e). These results indicate that the Ser 246-Pro motif of β-catenin is crucial for Pin1 to affect the subcellular localization of β-catenin.

Correlation between nuclear localization of β-catenin and overexpression of Pin1 in human breast-cancer tissues. To examine further whether Pin1 expression was correlated with the subcellular localization of β-catenin *in vivo*, we determined both the expression of Pin1 and the subcellular localization of β-catenin in 40 primary human breast-tumour tissues by immunohistochemical staining. As shown previously (ref. 27 and Fig. 4c), Pin1 is overexpressed to various degrees in breast-cancer tissues. Interestingly, β-catenin accumulated in the nuclear/cytoplasmic fraction in tumour tissues containing high levels of Pin1, whereas the localization of β-catenin was primarily at membranes in tumour tissues containing low levels of Pin1 (Fig. 8, Table 2 [AUTHOR: O.K. to cite what was Fig. 8b here?]). In 40 tumour tissues examined there was a significant correlation between Pin1 expression and the subcellular localization of β-catenin, as determined by the Spearman rank correlation test ($P < 0.01$). These results further support the notion that Pin1 is important in the regulation of β-catenin and strengthen the significance of Pin1 overexpression in the activation of β-catenin in human breast cancer.

Discussion

We have recently found that Pin1 is drastically overexpressed in several human cancer tissues, including breast and prostate cancers, and that Pin1 binds phosphorylated c-Jun and increases its ability to activate the cyclin D1 promoter via the activator protein-1 (AP-1) site[27]. These findings indicate that overexpression of Pin1 might contribute to oncogenesis via the modulation of gene expression. To examine this possibility further, we performed gene expression profiling with the differential display method. Interestingly, four of twelve upregulated genes identified are target genes downstream of that for β-catenin, which have been confirmed by northern and western analyses. Furthermore, Pin1 activates cyclin D1 and TOPFLASH promoters via the TCF sites. Moreover, the overexpression or depletion of Pin1 levels in cell lines significantly alters the levels of β-catenin by affecting its protein stability. The significance of this observation *in vitro* is substantiated by the findings that upregulation of Pin1 in breast cancer is strongly correlated with β-catenin levels in the tumours, whereas β-catenin levels were markedly decreased in tissues in Pin1 knockout mice. These results demonstrate that Pin1 regulates the stabilization of β-catenin under both physiological and pathological conditions.

The identification of the gene encoding cyclin D1 as one of the Pin1-induced genes in the differential display screen has confirmed our early findings that Pin1 is correlated with the overexpression of cyclin D1 in human breast cancer and activates the cyclin D1 promoter *in vitro*[27]. Pin1 can activate the cyclin D1 promoter via the AP-1 site in collaboration with the Ras signalling pathway[27]. Our present studies have further demonstrated that, as Pin1 concentrations increase, Pin1 can also activate the cyclin D1 promoter via the TCF sites through the activation of the β-catenin signalling pathway. These results indicate that Pin1 might regulate the cyclin D1 promoter via different pathways, on the basis of the gene reporter assays. This is consistent with the findings that Pin1 levels are correlated with cyclin D1 levels in breast cancer[27]. Recently, we also found that mice lacking Pin1 demonstrate phenotypes remarkably similar to those in cyclin-D1-deficent mice (Y.-C.L., [AUTHOR: please give all names (with initials)], unpublished results). It has been shown that high β-catenin activity is significantly correlated with cyclin D1 expression and poor prognosis, and is a strong and independent prognostic factor for human breast cancer[24]. Our findings that Pin1 is correlated with β-catenin in breast-cancer tissues indicate that Pin1 might be a potential prognostic marker.

Pin1-catalysed prolyl isomerization can induce a conformational change in proteins and thereby affects protein activity, protein dephosphorylation and/or protein–protein interactions[28–30,32–35]. Given that β-catenin contains three Ser-Pro motifs, it might be a Pin1 substrate. Indeed, Pin1 binds to β-catenin *in vivo* and *in vitro*, but only after it has been phosphorylated. The binding site has been further mapped to the pSer 246-Pro motif located at the centre of Armadillo repeats; the surrounding sequence is consistent with the Pin1 binding specificity[30]. Importantly, Pin1 selectively blocks the interaction between β-catenin and APC. Furthermore, Pin1 increases an accumulation of β-catenin in the nucleus and activates the transcription of target genes downstream of that encoding β-catenin. These results indicate that Pin1 not only binds phosphorylated β-catenin and inhibits its interaction with APC, but also decreases β-catenin turnover and increases its nuclear translocation, resulting in the upregulation of target genes downstreamof that for β-catenin.

Recent studies on the crystal structure of the β-catenin complexes and the role of APC in a nuclear–cytoplasmic shuttling might provide an explanation of why Pin1 affects the interaction between β-catenin and APC and the accumulation of β-catenin in the nucleus. APC is a nuclear–cytoplasmic shuttling protein that can export nuclear β-catenin to the cytoplasm for degradation[20,22,43]. Interestingly, in contrast to two other Ser-Pro motifs that are buried in the helices in β-catenin, the Pin1-binding Ser 246-Pro motif is located at an exposed loop region between the two helices at the third Armadillo repeat (Supplementary Information, Fig. S1a)[41,42]. Importantly, this Ser 246-Pro motif is next to the binding site for APC, and mutations of Phe^{253} and Phe^{293} in β-catenin completely abolish their ability to bind APC (Supplementary Information, Fig. S1b)[41,42]. We therefore propose that Pin1 would bind and isomerize the pSer 246-Pro peptide bond in β-catenin, which would affect its ability to bind APC, thereby regulating the turnover and subcellular localization of β-catenin. Consistent with this notion is the observation that disruption of the ability of Pin1 either to bind or to isomerize the pSer/Thr-Pro motifs abolishes the ability of Pin1 to induce the translocation of β-catenin to the nucleus and to activate the β-catenin-dependent transcription. Thus, our results indicate that Pin1-dependent prolyl isomerization might be a novel mechanism for regulating the β-catenin and APC interaction.

In summary, our results show that overexpression of Pin1 contributes to the upregulation of β-catenin in tumours such as breast cancer, where β-catenin is upregulated in the absence of a mutation in APC or β-catenin. Because the inhibition of the enzymatic activity of Pin1 triggers tumour cells to enter apoptosis, inhibition of upregulated Pin1 might offer a novel anti-cancer strategy. □

Methods

Vectors.

pBS-β-catenin and pCDNA-β-catenin/Myc/His-tag were gifts from Dr S. Hatakeyama, cyclin D1 promoter constructs were gifts from R. Pestell, TOPFLASH and FOPFLAS [AUTHOR: I'FOPFLASH'—see the text] were a gift from Xi He, and pCDNA/Myc-dominant-negative-TCF-4 was a gift from Dr B. Vogelstein. β-Catenin deletion and site-directed mutants were made by PCR with *Pfu* DNA polymerase (Stratagene) and inserted into pCDNA3.1 and pDS-Red-C1 vectors.

Differential display.

Total RNA was extracted from Pin1-induced or non-induced MCF-7 cells by using the Tet-Off Gene Expression System (Clontech) in accordance with the manufacturer's protocol. Cells were collected at 24 h after induction and total RNA was extracted with TRIzol reagent (Gibco BRL). Fifty micrograms of total RNA was used for constructing the cDNA library for differential display screening as described previously[37,38], with minor modifications. cDNA fragments with two different adapters on both sides were amplified by PCR in the presence of [^{32}P]dCTP with adapter-specific primer sets. The amplified cDNAs were separated on a 6% polyacrylamide gel with urea and detected by autoradiography with X-ray film exposed overnight. By displaying about 10,000 cDNA fragments from Pin1-overexpressing breast-cancer MCF-7 cells and control MCF-7 cells, 48 obviously different bands were recovered and re-amplified by PCR with the same primer set and cloned into pUC118 vector. Recombinant plasmid DNAs were sequenced with Big Dye terminator kits (PE Applied Biosystems, Branchburg, New Jersey) with an ABI 377XL automated sequencer (PE Applied Biosystems).

Northern blot analysis.

Eight micrograms of total RNA was separated on a 1.2% agarose gel containing 0.66 M formaldehyde and transferred to a Hybond-N$^+$ membrane (Amersham) in accordance with the manufacturer's protocol. The filters were baked at 80 °C for 2 h. The cDNAs were labelled with [^{32}P]dCTP by using a Megaprime DNA-labelling System (Amersham Pharmacia). Membrane filters were hybridized with labelled probes in buffer (40% deionized formamide, 4 × SSC, 10% dextran sulphate, 1 × Denhardt's solution, 40 μg ml^{-1} salmon-sperm DNA, 0.1% SDS, 20 mM Tris-HCl, pH 7.5) at 42 °C for 16 h. The filters were washed twice at room temperature for 15 min each, and once for 30 min at 56 °C with 2 × SSC containing 0.1% SDS, then exposed to films.

GST pulldown assay, immunoprecipitation and immunoblotting analyses.

Cells were arrested at the G1/S phase or the mitotic phase, as described previously[33], and β-catenin and its mutants were translated *in vitro* with the TNT coupled transcription/translation kit (Promega) in the presence of 8 μCi [^{35}S]methionine. They were then incubated in *Xenopus* extracts, as described[33]. Cell lysates, or proteins translated *in vitro*, were incubated with 20 μl agarose beads containing GST–Pin1 or GST at 4 °C for 2 h, as described previously[33,35]. The precipitated proteins were washed with wash buffer containing 1% Triton X-100 and subjected to SDS–PAGE.

For immunoprecipitation, cells were harvested at 24 h after transfection and lysed with Nonidet P40 lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 μg ml^{-1} leupeptin, 1.0 μg ml^{-1} pepstatin, 0.2 mM PMSF). Cell lysates were incubated for 1 h with Protein A/G–Sepharose/mouse IgG complexes. The supernatant fraction was recovered and immunoprecipitated with 2 μg anti-APC antibody (Ab-5; Oncogene Research) or anti-β-catenin antibody (Transduction Laboratories) and 30 μl Protein A/G–Sepharose. After being washed three times with lysis buffer, pellets were resuspended in 2 × Laemmli sample buffer and then analysed by SDS–PAGE. Membranes were immunoblotted with anti-β-catenin antibody, anti-GSK-3β antibody (Transduction Laboratories), anti-APC (Ab-1), anti-lamin B or anti-EB1 antibody (Oncogene Research).

Pulse–chase analysis.

Cells were grown in 60-mm dishes to 60% confluence in normal growth medium. After 24 h of transfection, cells were washed twice with HEPES-buffered saline solution [AUTHOR: O.K.?] (HBSS) and

pulse-labelled for 1 h in 1 ml methionine- and glutamine-free minimal essential medium (Gibco BRL) supplemented with 4 mM glutamine, 10% dialysed fetal calf serum and 100 μCi [^{35}S]methionine. Labelled cells were washed twice with HBSS and rinsed with normal growth medium. Cells were harvested at various time points and subjected to immunoprecipitation with β-catenin, followed by SDS–PAGE.

Gene reporter assay.

Approximately 60%-confluent cells were transfected in triplicate in 12-well dishes with Superfect (Qiagen). Gene reporter gene assays were performed with the Dual-Luciferase reporter assay system (Promega) at 24–30 h after transfection, as described previously[27]. pRL-TK (Promega) was used as an internal control for transfection efficiency. All results are expressed as means ± s.d. for independent triplicate cultures.

Immunostaining and cell fractionation experiments.

Cells were transfected with the indicated plasmids (1 μg of GFP vector, GFP–Pin1, GFP–Pin1^{WW*A} or GFP–Pin1^{K63A} in Fig. 7a, b; 0.2 μg of RFP–β-catenin or RFP–β-catenin^{S37AA} with 1 μg of GFP vector or GFP–Pin1 in Fig. 7e,f) and were fixed with 3.7% buffered formaldehyde for 5 min and stained for β-catenin protein with anti-β-catenin antibody. Staining with antibodies was performed as described previously[48,58]. Nuclei were revealed with 4′,6-diamidino-2-phenylindole (DAPI) staining for immunofluorescence microscopy. To isolate nuclear and cytoplasmic fractions, transfected cells were washed twice with cold PBS and lysed with hypotonic buffer (HEPES pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA). After incubation for 10 min at 4 °C, cells were treated with 0.5% Nonidet P-40 and centrifuged at 1,000 r.p.m. for 10 min. After recovery of supernatants representing cytoplasmic components, the pellets comprising nuclear extracts were washed once with hypotonic buffer and centrifuged at 1,500 r.p.m. for 5 min and lysed with 2 × Laemmli sample buffer.

Immunohistochemistry.

A breast-cancer array was purchased from Immugenex. Paraffin was removed from slides with xylene; slides were then hydrated with 100% and 75% ethanol and washed with water. The antigen recapture procedure was performed by boiling in a microwave oven for 10 min in 1 × antigen retreat citra [AUTHOR: what is 'antigen retreat citra'?] (Biogene). Slides were treated with PBS containing 5% goat serum and 0.1% Triton X-100 for blocking, and then with anti-Pin1 antibody or anti-β-catenin antibody at 4 °C in a humidified chamber for 12 h. After being washed with PBS, slides were incubated with biotinylated secondary antibody for 2 h. Immunohistochemical analysis was performed with a Vectastain ABC kit and DAB staining solution (Vector Laboratories, Burlingame, California).

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Supplementary information is available on *Nature Cell Biology*'s website (http://cellbio.nature.com). [CHECK wording with James]